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Semaphorin 3C guides MGE-derived cortical interneurons through the basal telencephalon

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“Life is a continuous process of welcoming and letting go, but I am keeping you forever in some of my hippocampal neurons… hope you like it.

Je amo Emilu.”

In memory of Emilia di Tommaso Aiello

Wendy Hellier.

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Chapter 1: Introduction

1.1 The cerebral cortex of the mammalian brain

Among mammals the cerebral cortex reaches its maximal complexity in humans, enabling us to have higher cognitive abilities like language, abstraction, and emotions. For this, an accurate balance between excitatory and inhibitory neurons is crucial with an assembly that is nothing less than a marvel of nature (Dehay and Kennedy 2007, Kennedy, Douglas et al. 2007). Defects and mist positioning of inhibitory cortical neurons has been linked with malfunctions like autism, epilepsy and schizophrenia (Ross and Walsh 2001, Powell, Campbell et al. 2003, Levitt, Eagleson et al. 2004, Cobos, Calcagnotto et al. 2005, Horike, Cai et al. 2005, Francis, Meyer et al. 2006, Friocourt, Poirier et al. 2006, Friocourt and Parnavelas 2010). Thus, the migration, positioning, and integration of cortical interneurons during development are crucial for proper cortical function.

Cortical neurons have been classified as inhibitory or excitatory in two populations: 80% originate in the proliferative zone of the pallium (cortex) and migrate radially into the developing layers (Fig. 1 a). These pyramidal neurons use glutamate as neurotransmitter and send constant messages to the organism. By themselves they would only generate an avalanche of excitation. The remaining 20 % correspond to inhibitory interneurons that originate in the subpallium (basal telencephalon). Cortical interneurons migrate tangentially over long distances to their final position in the developing neocortex, following distinct paths (Fig. 1 b, c). Interneurons express GABA (□-aminobutyric acid) as neurotransmitter and have far more functions than just the “inhibition” of other neurons. “For example, GABA transmission regulates synaptic integration, probability and timing of action potential generation, and plasticity in principal neurons. Furthermore, interneurons generate and maintain network oscillations, which provide the temporal structures that orchestrate the activities of neural ensembles” (Anderson, Kaznowski et al. 2002, Xu, Cobos et al. 2004, Buzsáki 2010, Molnar and Butt 2013).

Cortical GABAergic interneurons constitute one of the most diverse groups of cells in the central nervous system (Markram, Toledo-Rodriguez et al. 2004, Gelman, Griveau et al. 2011). Thus, comprehend different neuronal populations which can be distinguish due to its morphology, electrophysiology and the expression of specific molecular markers (Huang, Di

Cristo et al. 2007, Brown, Chen et al. 2011)). Interestingly, each subtype of cortical interneuron is specialized in targeting different pyramidal cells or other interneurons from specific layers and domains in the cortex (Marin and Rubenstein 2001, Xu, de la Cruz et al. 2003, Klausberger and Somogyi 2008, Marín, Valiente et al. 2010, Miyoshi and Fishell 2011, Marin 2013). All this interneuron specificity has been shown to be formed since early on with their progenitor cells in the basal telencephalon, by the regional and temporal expression of transcription factors, which impose a particular set of receptors and ligands (Kriegstein and Noctor 2004, Butt, Fuccillo et al. 2005, Flames, Pla et al. 2007, Batista-Brito, Rossignol et al. 2009, Chauvet, Cohen et al. 2009, Nobrega-Pereira and Marin 2009, van den Berghe, Stappers et al. 2013).

As shown in Figure 1, the basal telencephalon is divided into different transient domains, the lateral ganglionic eminences (LGE), the medial ganglionic eminences (MGE), the caudal ganglionic eminences (CGE) described as a caudal extension of the LGE and the MGE (Flames, Pla et al. 2007) and ventrally the preoptic area (POA). The MGE and the CGE are the major sites of interneuron genesis, producing 90% of the cortical GABAergic interneurons (Nery, Fishell et al. 2002, Xu, Cobos et al. 2004, Butt, Fuccillo et al. 2005, Wonders, Welagen et al. 2009, Miyoshi, Hjerling-Leffler et al. 2010, Gelman, Griveau et al. 2011, Marin 2013). Recently, the POA has been reported as the source of the remaining 10 % of cortical interneurons (Gelman, Griveau et al. 2011, Zimmer, Rudolph et al. 2011).

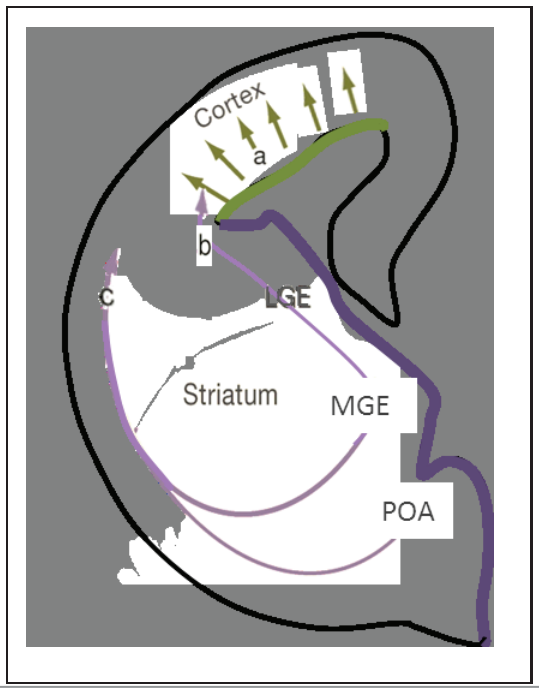


Figure 1. Radial and tangential migration towards the neocortex. Illustration of a coronal brain section at embryonic day 14. (a) Projection neurons migrate radially from the pallial ventricular zone into the neocortex. Cortical GABAergic interneurons originate from subpallial structures, the MGE, CGE (not shown) and POA and migrate tangentially through (b) the deep migratory stream or (c) the superficial migratory stream towards the neocortex. MGE, Medial ganglionic eminence; LGE, Lateral ganglionic eminence; CGE, Caudal ganglionic eminence; POA, Preoptic area. Modified from Ayala et al. (2007)

1.2 Cortical interneuron migration

The time span of neocortex development varies among species. In the mouse, corticogenesis occurs from embryonic day 11 (E11) to embryonic day 19 (E19). A peak of cortical interneuron migration has been reported around the embryonic day E14, which coincide with the highest production of interneurons in the medial ganglionic eminences (Corbin and Butt 2011). For this reason, in the mouse brain, E14 is the embryonic age commonly used for studying the cortical interneuron migration.

Cortical interneurons migrate along different paths, from the basal to the dorsal telencephalon, reaching their final position in the developing neocortex. Such process is usually named “Tangential migration” (Marin and Rubenstein 2001, Arber and Li 2013). As shown in Figure 1, cortical interneurons that were born in the POA predominantly migrate parallel to the surface and ventrally to the striatum along the superficial migratory stream (SMS). They enter the cortex in the marginal zone. In contrast, cortical interneurons from the MGE migrate along the deep migratory stream (DMS) along the ventricular zone of the basal telencephalon and dorsally of the striatum. These interneurons enter the SVZ of the dorsal telencephalon (Marin, Yaron et al. 2001, Zimmer, Rudolph et al. 2011, Marin 2013).

It is intriguing how the precise guidance of GABAergic neurons is orchestrated, since they originate at a considerable distance from their targets. Nowadays it is widely accepted that cortical interneuron migration is achieved through cell intrinsic programs, due to the expression of specific receptors and ligands. And also those receptors and ligands conform and respond to extracellular cues. The whole process is mediated by numerous intracellular signaling pathways that rearrange cytoskeletal components of the interneurons (Nobrega-Pereira, Kessaris et al. 2008, Nobrega-Pereira and Marin 2009).

Extracellular molecules work in conjunction to orientate cortical interneurons towards their final target (Fig. 2). Guidance molecules have been classified in motogenic, repellent or attractive. Motogenic cues stimulate the movement of the interneurons. Some examples are the brain-derived neurotrophic factor (BDNF) and hepatocyte growth factor (HGF) expressed in the dorsal border in the ventricular zone of the eminences (Fig. 2). More recently EphA4/ephrinA2 interactions were described to promote the movement of migrating cortical interneurons. Both are expressed in the VZ of the medial and lateral ganglionic eminences, with overlapping patterns, as shown in Figure 2 (Steinecke In submission).

Attractive molecules, as the name indicates, guide cortical interneuron towards them. One example is the protein Neuregulin-1 (Nrg1), and its receptors ErbB4. ErbB4 receptor is expressed by tangentially migrating neurons and co-localized with the interneuron marker DLX2 (Yau, Wang et al. 2003). There are two isoforms of Neuregulin-1, one is membrane bound Nrg1-CRD which contains an extracellular cysteine rich domain (CRD) and act as short-range attractant for interneurons. This isoform is expressed by lateral ganglionic eminence (LGE) derived cells, creating a corridor where cortical interneurons pass over the LGE towards the cortex. The second isoform is secreted Ig-Nrg1 that contains an extracellular immunoglobulin (Ig)-like domain, and is express along the ventricular zone (VZ) of the neocortex, proven to be a long-range attractant for interneurons (Flames, Long et al. 2004). Another guidance molecule is the secreted chemokine Cxcl12 with it receptor CXCR4 and scavenger receptor CXCR7. Both receptors CXCR7 and CXCR4 are coexpressed in migrating cortical interneuorns derived from the MGE (Sanchez-Alcaniz, Haege et al. 2011). An interesting feature of Cxcl12 is its binding to the scavenger receptor CXCR7. The naming of the scavenger receptor CXCR7 is based on its function of “cleaning” or internalized exceeding Cxcl12. The elimination of Cxcl12 through CXCR7 allows a more precise CXCR4/Cxcl12 signaling (Sanchez-Alcaniz, Haege et al. 2011, Wang, Li et al. 2011). Finally, CXCR4/Cxcl12 interaction channels interneurons that reached the neocortex through two streams: one along the marginal zone (MZ) and the second one over the subventricular zone (SVZ) (Fig. 2) (Marín, Valiente et al. 2010, Marin 2013).

Additionally, cortical interneurons avoid repellent molecules. Among repellent cues are some proteins of the ephrin family. The ephrins are membrane bound proteins which receptors are Eph tyrosine kinases. For example, neurons derived from the MGE expressed EphA4 receptors and co-localizedd with the early cortical interneuron marker calbindin (Zimmer, Garcez et al. 2008). EphA4 interacts with different ephrin ligands, channeling migrating cortical interneurons in the appropriate migratory paths. Specifically, ephrinA5, ephrinA3 and recently reported ephrinB3, channel interneurons derived from the VZ/SVZ of the MGE into the deep migratory route by repulsive interactions thought its receptor EphA4 (Zimmer, Garcez et al. 2008, Rudolph, Zimmer et al. 2010). As shown in Figure 2, ephrinA5 is expressed in the ventricular zone of the subpallium and in the POA, displacing MGE-derived interneurons from the place of origin and avoiding the invasion of the POA. On the other hand, ephrinA3 is expressed in the striatum and in the cortical plate of the neocortex,

preventing interneurons from innervating the striatum and channeling interneurons towards the marginal zone of the neocortex. In the case of EphA4/ephrinB3, their interaction induces a repellent response in MGE-derived interneurons, preventing them from following the deep migratory path (Zimmer, Rudolph et al. 2011, Steinecke In submission).

For Ephrins bidirectional signaling has been described. Meaning, that receptor/ligand interaction could also trigger signaling in the cell that bears the ligand. The interaction induced by a receptor acting as a ligand is called “reverse signaling”. In the case of, EphA4 not only act as a receptor, but also induce a signal cascade in ephrinB3-expressing cells. EphA4/ephrinB3 reverse signaling repels interneurons generated in the POA and orientates them through the superficial migratory stream (Zimmer, Rudolph et al. 2011). As a result, EphA4/ephrinB3 bidirectional interactions restrict intermingling of the MGE- and POA-derived cortical interneurons sorting them in the superficial and deep migratory streams (Zimmer, Rudolph et al. 2011).

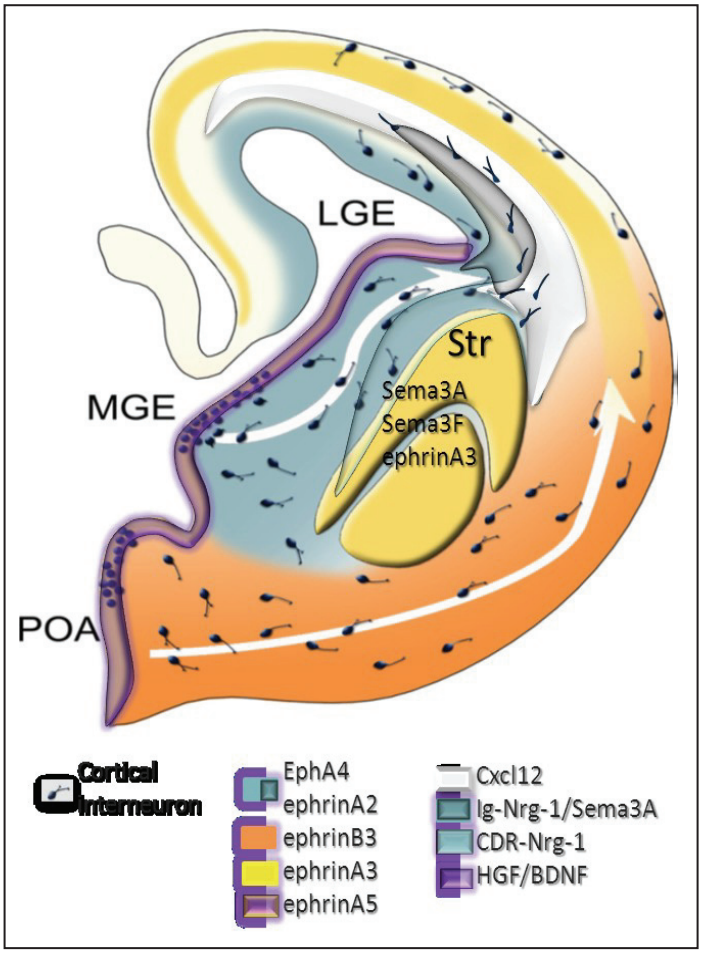


Figure 2. Model for the segregation of POA- and MGE-derived interneurons into the superficial and deep migratory stream. Drawing of a coronal brain slice at E14. MGE-derived interneurons expressed EphA4 and migrate along the deep migratory route. EphA4/ephrinA5 interactions expel interneurons from their place of origin in the ventricular zone of the MGE, preventing ventral migration and thus invasion into the POA. Therefore EphA4/ephrinA5 interactions, directs cortical interneurons towards their target. The expression pattern of EphA4 in the VZ of the MGE and LGE overlaps with the spatial expression of ErbB4 and Cxcr4, receptors of Nrg1 and Cxcl12 respectively. ErbB4/Nrg1 interactions attract cortical interneurons through the deep migratory stream and neocortex (Flames et al., 2004; Wang et al., 2011). HGF, BDNF and ephrinA2/EphA4 interaction promotes the movement of the migrating cortical interneurons. In contrast, interneurons generated in the POA expressed ephrinB3 and traverse the superficial pathway. ephrinB3 binds to EphA4 and induces a repellent response in MGE-derived interneurons. In addition, EphA4 also acts as a ligand for ephrinB3, such "reverse signaling" repels POA-derived interneurons. Thus, cell–cell contact mediated bidirectional ephrinB3/EphA4 signaling. Such signaling restricts intermingling of MGE- and POA- derived interneurons. Thus, bidirectional repulsive effects sort interneurons subtypes in the deep and superficial migratory stream. Sema3A and Sema3F kept Nrp1- Nrp2- bearing interneurons away from the striatum. ephrinA3 also is involved in keeping the developing striatum and the cortical plate of the neocortex free of cortical interneurons. Once in the neocortex, chemokine Cxcr4/Cxcl12 attracts interneurons through two streams in the marginal zone (MZ) and the subventricular zone (SVZ) of the neocortex. In contrast, Sema3A expressed in the ventricular zone and ephrinA3 expressed in the cortical plate might repel migrating interneurons. MGE, Medial ganglionic eminence; LGE, Lateral ganglionic eminence; POA, Preoptic area; Str, Striatum; E, Embryonic day. Modified from Rudolph et al. (2010); Zimmer et al. (2011) and Marin et al. (2011).

1.3 Semaphorins

Semaphorins are described as guidance molecules with major roles in immune function, cardiac growth and vascular development (Roth, Koncina et al. 2009). Semaphorins are also well known for axonal guidance during the development of the nervous system in vertebrates. Every Semaphorin is characterized by the expression of a specific region called the “sema domain” of about 500 amino acids. Currently, 27 Semaphorin proteins have been identified and grouped into 8 classes on the basis of structural and amino acid sequence similarities (Cell 1999). Class 1 and 2 Semaphorins are proteins present in invertebrates, class 3 to 7 Semaphorins are present in vertebrates and class 8 Semaphorins are present in viruses. Only Semaphorins from the classes 2, 3 and 8 are secreted proteins, the remaining Semaphorins are membrane bound. The main receptors for Semaphorins are the Plexins. There exist 9 Plexins in vertebrates: PlexinA1–PlexinA4, PlexinB1–PlexinB3, PlexinC1 and PlexinD1 (Perala, Sariola et al. 2012). It is thought that, in most cases Semaphorins signal through specific receptor complexes that vary among each Semaphorin molecule.

One of the most versatile Semaphorin classes is number 3, composed by 7 molecules, ranging from Sema3A to Sema3G. Being secreted proteins, Sema3s gradients have been shown to be a key factor that modulates the chemotaxis of cortical axons. Specifically, research provided evidence that increasing gradients of Sema3C wired cortical axons towards its target in the neocortex, whereas Sema3A gradients repelled them (Bagnard, Lohrum et al. 1998, Ruediger, Zimmer et al. 2013). Another interesting feature of Sema3s is that they use Neuropilins as co-receptors. Neuropilins have a small cytoplasmatic domain. Therefore, they need Plexin molecules to transduce their signals across a cell membrane (Fig. 3).

Two types of Neuropilins have been described; Neuropilin 1 (Nrp1) and Neuropilin 2 (Nrp2) which form dimers with different affinity to each types of Sema3s. For example, Nrp1 homodimers have high affinity to Sema3A, Nrp2 homodimers have a high affinity for Sema3F, and Nrp1/ Nrp2 have a high affinity for Sema3C (Chen, Chedotal et al. 1997, He and Tessier-Lavigne 1997, Kolodkin, Levengood et al. 1997, Takahashi, Fournier et al. 1999, Gu, Rodriguez et al. 2003). On the same way, each type of Neuropilin dimer will form a receptor complex with a specific type of Plexin. The organization of these complexes likely provides specificity for binding and transducing signals from different Class 3 Semaphorins. Such

amounts of possible combinations make it interesting and difficult for researchers to determine the specific co-receptors for each type of Sema3s. (He and Tessier-Lavigne 1997, Castellani and Rougon 2002, Pasterkamp 2012, Sharma, Verhaagen et al. 2012).

Furthermore, as shown in Figure 4, Nrp1 and Nrp2 are express on MGE-derived cortical interneurons, segregated through the deep and superficial migratory streams. Therefore, Neuropilin/Sema3s interaction in the basal telencephalon seems to orientate interneurons in the deep and superficial migratory pathways. Specifically, Nrp1 is highly expressed in the SVZ of the neocortex and the POA. Only a small percentage, between 15-20 % of the MGE-derived cells express Nrp1, being restricted to the deep migratory stream. In contrast, Nrp2 is expressed in the POA and ventrally to the striatum, demarcating the surface of the superficial migratory stream (Nobrega-Pereira and Marin 2009, Zimmer, Rudolph et al. 2011, Marin 2013).

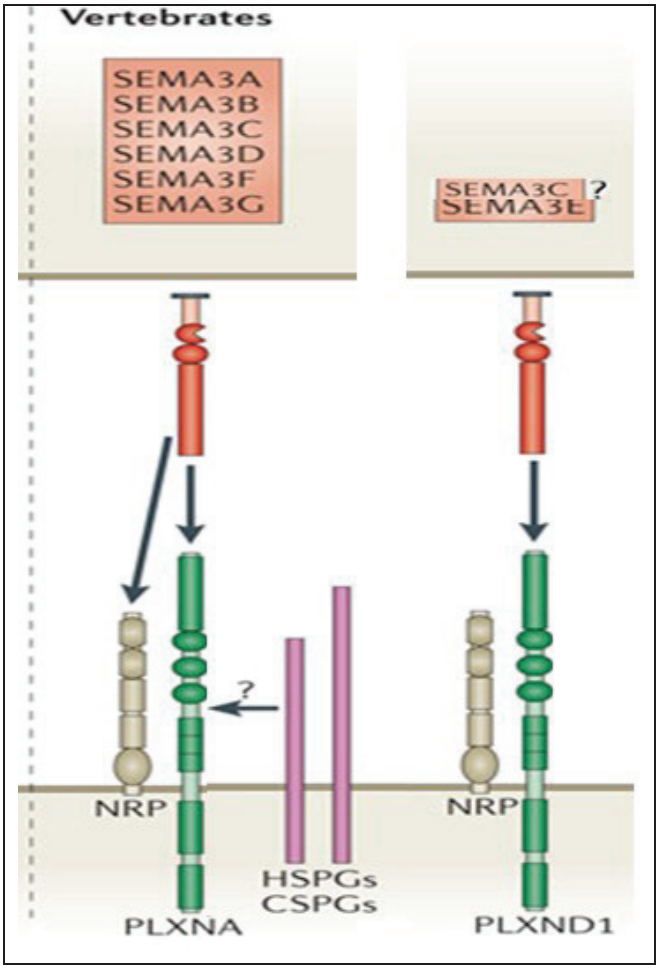


Figure 3. Class 3 Semaphorins and their receptor complexes. Generally, Semaphorins interact with Plexins receptors. In vertebrates, besides Plexins, Sema3s also requires Neuropilin binding (Fujisawa 2004). Chondroitin sulphate Proteoglycans (CSPGs) were proven to modulate Sema3A signaling(Zimmer, Schanuel et al. 2010). CSPGs interaction with other Sema3s remains to be elucidated. Neuropilin (NRP); Plexin (PLXN); Chondroitin sulphate proteoglycans (CSPGs); Heparan sulphate proteoglycans (HSPGs); Modified from (Pasterkamp and Giger 2009, Pasterkamp 2012).

Semaphorin 3A was the first Semaphorin protein described in vertebrates, originally was named “collapsin”, referring to its transitory collapse effect on axonal growth cones (Luo, Raible et al. 1993). Axonal growth cones screen their environment in order to search their target regions and respond to chemotactic cues. Therefore, axonal growth cones have been widely used to test the effect of guidance molecules. Another interesting feature of Sema3A was that channels cortical axons into the neocortex through a repulsive effect (Bagnard, Lohrum et al. 1998, Ruediger, Zimmer et al. 2013).

Several studies have been performed regarding growth cones of migrating axons. Such studies described that axonal growth cones generally showed spread morphology and an enlarged morphology during pausing behavior. On contrary, during advance, growth cones have been described as a “bullet like morphology” being small and streamlined, whereas growth cones seemed completely absent during collapse (Mason and Wang 1997).

During migration, cortical interneurons present a neurite (also called process) that can bifurcate, elongate and/or retract. The structure at the end of the neuronal process are named “growth cone” making allusion to its resemblances with axonal growth cones. Even though the morphology of axonal growth cones seem similar to the structure of migrating cortical interneurons, further studies are needed to elucidate its functions and responses to guidance cues. Once clarified, growth cones from migrating interneurons could bring new insights about how migrating interneurons response to guidance cues. For example, currently it remains to be elucidated if Sema3A affect the growth cones of migrating cortical interneurons “collapsing” them on a similar way as Sema3A affects axonal growth cones.

The expression patterns of different members of the class 3 Semaphorin (Sema3s), including Sema3A, Sema3C, Sema3E and Sema3F are already described in the cerebral cortex of the developing mouse brain (Bagnard, Lohrum et al. 1998, Skaliora, Singer et al. 1998, Bagnard, Thomasset et al. 2000, Ruediger, Zimmer et al. 2013). Thus far, Sema3C has been the only Sema3s protein shown to attract cortical axons to the subventricular zone of the neocortex. The attractive effect of Sema3C only occurs on increasing concentrations of the protein and do not seems to affect axonal growth cones (Bagnard, Lohrum et al. 1998, Ruediger, Zimmer et al. 2013). Possible Sema3C binding partners have been suggested to be a complex of Nrp1 and Nrp2 heterodimers that binds to PlexinA1 or PlexinD1 (Kruger, Aurandt et al. 2005, Zhou, Gunput et al. 2008).

Regarding Class III Semaphorin and the migration of cortical interneurons, only the effects of Sema3A and Sema3F have been studied (Marin, Yaron et al. 2001, Tamamaki, Fujimori et al. 2003, Zimmer, Schanuel et al. 2010). Such studies indicated a repulsive effect from, Sema3A and Sema3F over migrating cortical interneurons. Furthermore, it has been suggested that the repellent effect of Sema3A is enhanced by Chondroitin sulphate Proteoglycans (CSPGs) (Fig. 3) (Zimmer, Schanuel et al. 2010). Sema3A and Sema3F effects over migrating cortical interneurons are similar to the effects described over cortical axons. In contrast, so far, no information is available regarding the effect of Sema3C on migrating cortical interneurons. As shown in Figure 3, Sema3C sharply demarcates the pallial/subpallial border and the intracortical pathway of interneurons migrating developed in the subventricular zone. Also Nrp1 and Nrp2, possible co-receptors of Sema3C, present complementary expression patterns (Fig. 4) Therefore, it would be interesting to test if Sema3C has an effect on migrating cortical interneurons. Even more, since mainly repellent guidance cues have been described, it would be relevant to examine if Sema3C exert an attractive effect, as shown already with cortical axons. For this reason, this study examined the possible role of Sema3C on the migratory behavior of MGE-derived cortical interneurons through different in vitro assays.

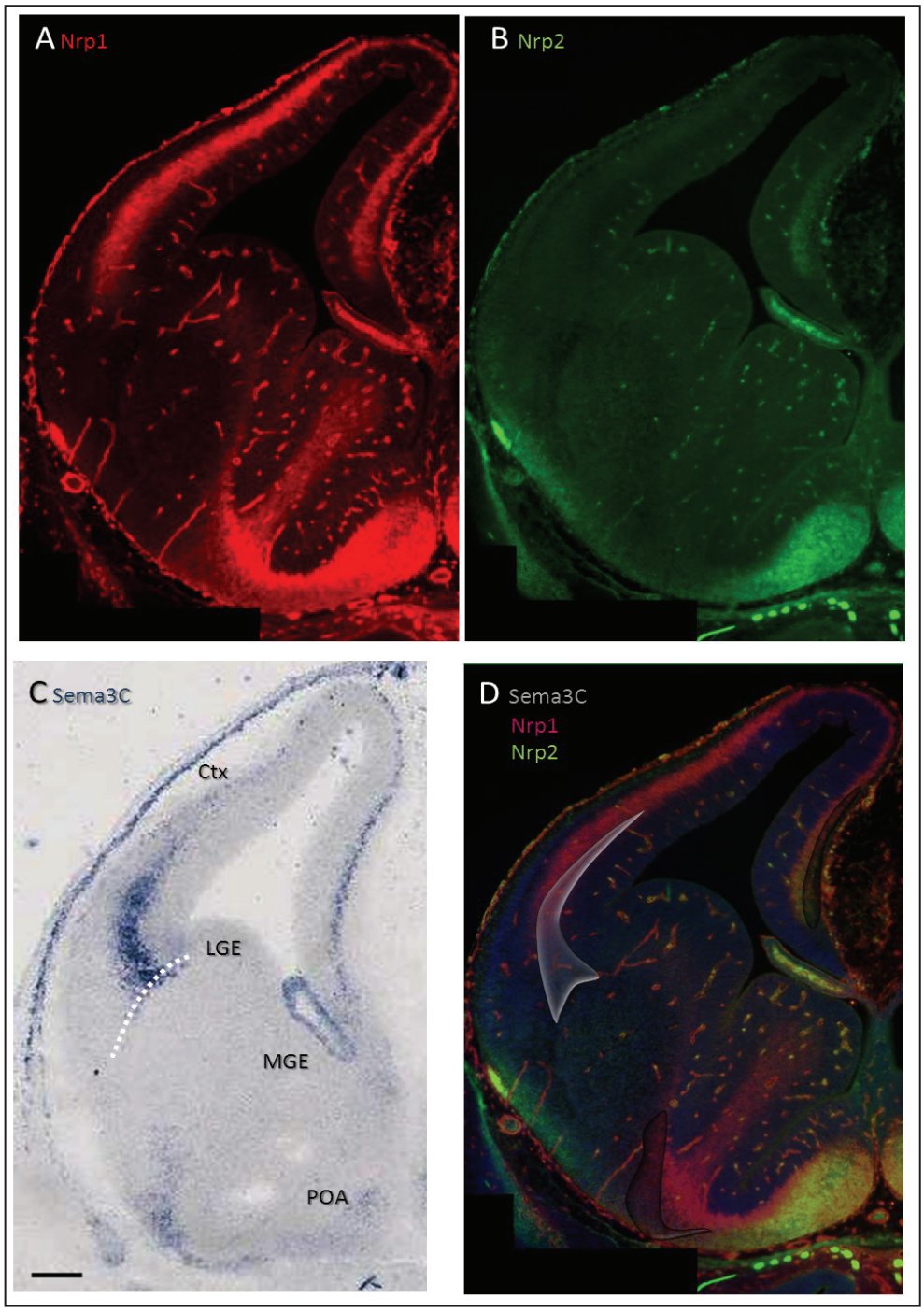


Figure 4. Complementary expression of Sema3C and its possible receptors Nrp1 and Nrp2. Coronal brain sections at E14.5. Immunohisto-chemistry on cryo slices against (A) Nrp1 shows expression in the cortical SVZ and IMZ, as well as in the MGE and POA. Staining against (B) Nrp2 shows an expression in the superficial migratory stream, ventrally to the striatum and in the POA. (C) An in situ hybridization shows Sema3C expression in the intermediate and subventricular zone of the neocortex. White dotted line shows part of the pallial/subpallial border. (D) Merged expression of Nrp1 (green) and Nrp2 (red) receptors with scheme showing the expression pattern of Sema3C (gray). Nrp1, Neuropilin 1; Nrp2, Neuropilin 2; Ctx, Cortex; IMZ, intermediate zone; SVZ, Subventricular zone; MGE, Medial ganglionic eminence; LGE, Lateral ganglionic eminence; POA, Preoptic area; E, Embryonic day. Scale bar: 250µm. Modified from Rudolph and Barchmann (Data not publish).

Chapter 2: Materials and Methods (Experimental procedures)

2.1 Materials

2.1.1 Mice strains

For the experiments, time pregnant mice of the strain C57BL/6 and NOR were used. The day of insemination was considered as embryonic day 1 (E1). Mice were bred and maintained under standard conditions with access to food and water ad libitum on a 12 hours (h) light/dark cycle. All animal procedures were performed in accordance with institutional regulations of Friedrich Schiller University of Jena, Germany.

2.1.2 Cell lines

As stable cell lines, Human Embryonic Kidney cells (HEK - 293), were used. These expressed either recombinant Semaphorin 3A (Sema3A) or Semaphorin 3C (Sema3C), both Alkaline Phosphatase tagged (-AP) and resistant to Geneticin, (Bagnard, Lohrum et al. 1998). Therefore, after thawing and making the first passage, the cells were kept in culture medium with 0.5 % Geneticin and minimum two more passages were performed before usage. Untransfected HEK cells were used as control.

In the present work, “control HEK” referred to the untransfected HEK cell line, and regarding the stable HEK cell line secreting recombinant Semaphorin 3A-AP or Semaphorin 3C-AP, are named “Sema3C-AP HEK” or “Sema3A-AP HEK” respectively.

2.1.3 Reagents, appliances, buffer solutions and media were described in the Appendix.

2.2 Cell biological methods

2.2.1 Sterile work

Cell culture, primary culture and assays were performed under sterile conditions in a laminar flow bench. Surfaces were cleaned with 70 % Ethanol. All buffer solutions, media, equipment and other utilities used were sterilized. Required dissecting tools (scalpel, tweezers, and forceps) were sterilized in a hot beat at 260 °C for 10 s. The workstations, incubators, buffer, media and reaction vessels for primary culture and cell culture were strictly separated reducing the risk of mycoplasma infection on the cell lines.

2.2.2 Incubation conditions

For the cell culture and primary cell culture separate incubators were used, the conditions kept were: 90 % humidity, 37 °C temperature and 5 % CO2.

2.2.3 Primary culture

Timed pregnant mice were anesthetized using peritoneal injection of 10 % chloral hydrate. After absence of pain reflexes, the abdomen was disinfected with 70 % ethanol, opened and the uterine horns were taken. E14.5 embryos were removed from the amniotic sacs, the heads were extracted and the complete brain isolated for dissection (as detailed in Section 2.2.3.1 and Section 2.2.3.2). All further steps were carried out under sterile conditions, as described in Section 2.2.1.

2.2.3.1 Dissection of the medial ganglionic eminence (MGE)

The hemispheres were isolated from the neural tube (Fig. 5 A) and in each hemisphere, the lateral ends were removed with a scalpel (Fig. 5 B). This allowed to open the cortex (Fig. 5 C) and to isolate the area of interest (MGE indicated by the blue arrow, Fig. 5 C). The pia mater was removed to avoid fibroblast contamination in the culture. Once extracted, the MGEs were placed in ice-cold collection medium (HBSS/Glucose) and single cells were prepared (Section 2.2.3.3).

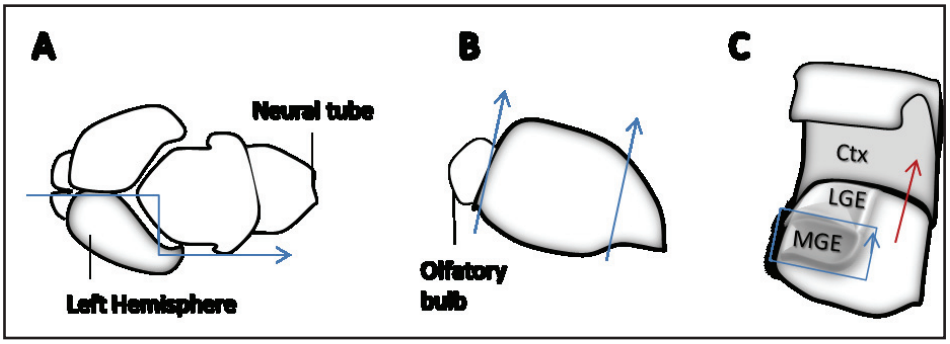


Figure 5. Procedure for E14.5 MGE dissections. Blue arrows indicate cutting areas. (A) Separation of hemispheres from a complete brain. (B) Lateral ends were removed from a hemisphere. (C) The cortex was opened as shows the red arrow, allowing the access to the medial ganglionic eminence. Ctx, Cortex; MGE, Medial ganglionic eminence; LGE, Lateral ganglionic eminence; E, Embryonic day.

2.2.3.2 Dissection of the ventricular and subventricular zone (VZ/SVZ) or intermediate zone (IMZ) of medial ganglionic eminences.

260 µm coronal brain slices were produced using a Tissue Chopper (McIlwain; Blue lines Fig. 6 A). Coronal sections were transferred to ice-cold GBSS/glucose. Slices with visible MGE were selected and the tissue of interest was extracted with scalpel and tweezers (Blue arrows Fig. 6 B). The isolated tissues were kept in ice-cold collection medium (HBSS/Glucose) for preparing single cells or explants (as detailed in Section 2.2.3.3 and Section 2.2.3.4, respectively).

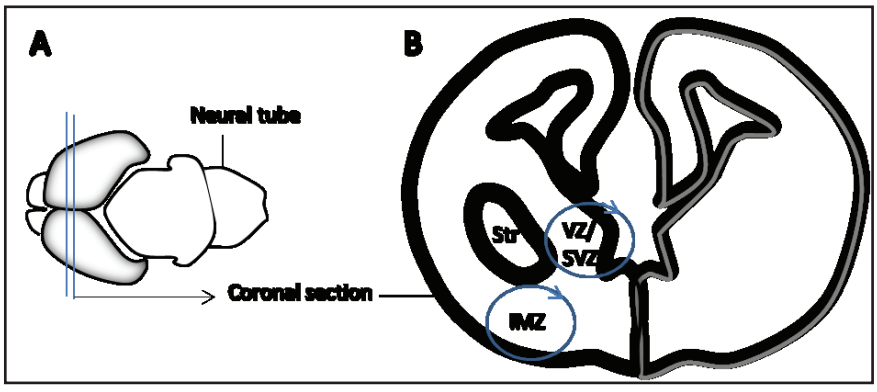


Figure 6. Guideline for the dissection of E14.5 VZ/SVZ or IMZ of the MGE. Blue arrows point at cut regions. (A) 260 µm coronal brain slices were obtained from a complete E14.5 brain. (B) Scheme of coronal section, with two hemispheres containing the ganglionic eminences, the blue arrow enclosed the VZ/SVZ or IMZ of the medial ganglionic eminence. Str, Striatum; VZ/SVZ, Ventricular/subventricular zone; IMZ, Intermediate zone of the medial ganglionic eminences; E, Embryonic day.

2.2.3.3 Preparation of dissociated cells

The tissue collected in ice-cold collection medium (HBSS/Glucose) was incubated with 0.25 % trypsin in water bath, at 37 °C for 17 minutes (min), (1 ml trypsin was added into 5 ml HBSS/Glucose). Next, the supernatant was removed. Afterwards, to stop the trypsin reaction, 1 ml of ice-cold culture medium containing fetal bovine serum (FBS) was added. The tissue was homogenized by repeatedly pipetting up and down with a rounded glass Pasteur pipette. Then, cell aggregates were excluded by filtering the cell suspension through nylon net (Mesh size: 120 µm). Cells were counted using a cell counter chamber and the density of the cell solution was adjusted to the plating concentration required. For all in vitro assays the cell concentration was 90.000 cells/100 µl culture media, applying 100 µl on each glass cover slip. Only for the Collapse assay a concentration of 80.000 cells/100µl culture media was used. Further seeding and culturing steps are described in Section "2.4 In vitro assays".

2.2.3.4 Preparation of explants

The isolated MGEs were shopped in ice-cold collection medium (HBSS/Glucose) using a Tissue Chopper (McIlwain) (200 x 200 µm2) or by hand with a scalpel. The explants obtained were placed in methylcellulose medium, separated by pipetting up and down and incubated for at least 1 h, allowing them to round. Further seeding and culturing steps are described in Section "2.4 In vitro assays".

2.2.4 Cell culture techniques

2.2.4.1 Thawing of cells

Vials containing HEK cells, stored in liquid nitrogen at -120 °C were removed from the liquid nitrogen, thawed in a water bath (37 °C) and transferred to 4 ml warm cell culture medium (37 °C). After centrifugation (4 °C, 800 x g, 5 min), the supernatant was removed and the cell pellet resuspended in 1 ml cell culture medium (37 °C) by pipetting up and down 10 times.

A passage was performed as soon as cells were confluent. It could take from 24 h up to 4 days culturing to obtain an 80 % confluent dish. HEK cells transfected to produce Sema3A-AP or Sema3C-AP have Geneticin resistance, therefore, after the first passage, 0.5 % Geneticin was added to the cell medium. To eliminate non-transfected cells after thawing, minimum three passages should be performed before carrying out any in vitro assays with them.

2.2.4.2 Passaging of cells

When 80 % cell confluency was reached, the medium was discarded, the cells were washed with 1 ml warm PBS (37 °C). 1 ml of 0.25 % trypsin was added and incubated for 3 min (room temperature). The trypsin reaction was stopped by adding 3 ml of FBS-containing warm medium (37 °C). Cells were detached from the Petri dish surface due to shaking and flushing. The cell suspension was collected and centrifuged (20 °C, 800 x g, 5 min). The supernatant was removed and the pellet resuspended in 1 ml warm cell culture medium (37 °C) by repeatedly pipetting up and down (around 10 times). Cells were seeded in a 10 cm Petri dish containing 10 ml warm medium (37 °C).

2.2.4.3 Freezing and storage of cells

For the conservation of the cell lines, 80 % confluent cells, between passage 4 and 8, were frozen. For freezing the cells, a normal passage was performed (Section 2.2.4.2), but after centrifugation, the cell pellet was resuspended in 1 ml freezing medium by pipetting up and down around 10 times. Freezing medium consist of 10 % dimethyl sulfoxide (DMSO) and 90 % FBS-containing cell culture medium. The cell suspension was transferred to a cryogenic vial. The cells were cooled down 1 °C per min using an isopropanol chamber placed at -80 °C. After 24 h, the cells were stored permanently in liquid nitrogen at -120 °C.

2.2.4.4 Production of Semaphorin conditioned media

The two stable HEK cell lines secrets Sema3A-AP or Sema3C-AP recombinant proteins directly into the culture media (Bagnard, Lohrum et al. 1998). Therefore, control conditioned medium, Sema3A-AP or Sema3C-AP conditioned media were obtained as already described (Castellani, Chédotal et al. 2000, Deng, Poudel et al. 2007).

First, 10 cm Petri dishes with 60 % confluent untransfected, Sema3A-AP or Sema3C-AP HEK cells, were rinsed with 1 ml warm PBS (37 °C). 6 ml warm serum-free medium (DMEM, 37 °C) was added to the cells and incubated for 3 days without Geneticin. In this time, the cells secreted the recombinant proteins into the medium. The media were collected and centrifuged to eliminate any cellular debris (20 °C, 800 x g, 5 min). The supernatant was directly concentrated, or stored at -20 °C for future concentration.

Second, the collected media were concentrated using a filter device (Millipore) that accumulates molecules bigger than 100 kDa. Since Sema3A-AP or Sema3C-AP has a

molecular weight around 150 kDa (Puschel, Adams et al. 1995, Adams, Lohrum et al. 1997), this filter device guaranteed Sema3A-AP or Sema3C-AP collection. Following the user manual, 12 ml of the collected medium was applied to the device and placed in the fixed rotor centrifuge oriented with the membrane facing up (5000 x g, 30 min). To recover the concentrated solution, a pipette was inserted into the bottom of the filter and the sample withdrawn immediately after centrifugation.

The concentrated solutions from control, Sema3A-AP or Sema3C-AP cultured media will be referred as “conditioned media” in the *in vitro* assay section 2.4. For calculating the concentration of each conditioned media, the initial volume added in the filter device was divided by the volume recovered after concentration. When used in *in vitro* experiments, conditioned media from control, Sema3A-AP or Sema3C-AP expressing HEK cells were diluted to a final tenfold concentration.

2.2.4.5 Production of cell aggregates for coculture assay

After performing a passage from 80 % confluent control, Sema3A-AP or Sema3C-AP HEK cells, the cell pellet were resuspended in 200 ml warm cell culture medium (37 °C). Using a Neubauer counting chamber, cells were counted and diluted to a concentration of 5 x 106 cells/200 µl. In the lid of a Petri dish, 20 µl drops of the cell solution were placed. The Petri dish was filled up with warm cell culture medium to keep humidity. Carefully, the lid was turned around and the Petri dish was closed with it. The cells were incubated overnight at 37 °C, 5 % CO2, and due to gravity concentrated in the middle of the drop forming aggregates. The procedure described is also known as "hanging drop".

After 1 DIV (day in vitro) the lid of the Petri dish was turned around, the cell aggregates were abraded from the drops with the aid of a spatula, and transferred in warm cell culture medium for further use. The dense parts of the cell aggregates were quartered and let rounded in the incubator for minimum 30 min.

2.3 Biochemical techniques

2.3.1 Confirmation of protein production by the HEK cells lines by western blot analysis

The production and secretion in the culture media of Sema3A-AP or Sema3C-AP recombinant proteins by the respective HEK stable cell lines were confirmed. Therefore, untransfected and transfected HEK cells lines were cultured during 5 and 8 h in 6 ml serum

free medium, 24 h in 6ml FBS-containing medium and 12 h under hanging drop conditions (Section 2.2.4.5) in 20 µl FBS-containing medium. After each incubation time, the culture media were carefully removed, and centrifuge to eliminate any cell debris and stored at -20 °C until the moment of use.

Thawed on ice, 10 µl of each medium was mixed in 100 µl cold STEN lysis buffer (Chapter 5: Reagents) with protein inhibitor cocktail (PI-Mix, Sigma). After 30 min incubation on ice, the suspension was centrifuged (4 °C, 13000 g, 10 min). The supernatant was transferred into a new reaction tube and the pellet discarded. Samples were kept on ice during all steps of preparation.

Once the electrophoresis module (BioRAD) was assembled and ready to use, the samples were set (20 µl sample medium mixed with 4 µl 6 X Laemmli sample buffer (Chapter 5: Reagents), incubated 5 min at 95 °C, separated by 8 % acrylamide SDS-PAGE (Merk) and electrotransferred onto a PVDF membrane (Millipore). Membranes were blocked in 0,2 % I-block in PBS with 0,1 % TW20 for 30 min at room temperature and then incubated with the primary antibody, against Sema3C or Sema3A (R&D systems), for 1 h at 4 °C. After washing in TBS-T (3 times, 10 min each), the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT and washed again in TBS-T (3 times, 10 min each). Signals were developed, excluded from light, with an enhance chemoluminescence system. Thus, 4 ml Solution A, 400 µl Solution B and 1,2 µl 30 % H_2O_2 was added on each membrane (Chapter 8, Section 8.2 Reagents). LAS4000 mini (Fuji) camera system and Image Quant program were used to acquire the blot pictures.

2.3.2 Photometric quantification of Semaphorin-AP activity

The photometric quantification is another approach for confirming the secretion of Semaphorin by the Sema3A-AP or Sema3C-AP HEK cell lines, using its alkaline phosphatase tag. This biochemical assay also tests the integrity and functionality of the alkaline phosphatase tag, and takes advantage of the fact that the AP enzyme is non-specific, and utilizes the non-biological substrate p-nitrophenyl phosphate to give p-nitrophenol upon hydrolysis. Because p-nitrophenyl phosphate is colorless, the formation of the yellow colored p-nitrophenylate can be used to measure the progress of the reaction. The color change is an indicator of the amount and functionality of the AP enzyme.

For the sample preparation, the untransfected, Sema3A-AP or Sema3C-AP HEK cell lines were incubated for 1, 3, 4, 5, and 24 h in culture medium. Each cell line was expected to secrete intrinsic proteins from HEK cells, used as control. In addition Sema3A-AP HEK or Sema3C-AP HEK cell lines were expected to also secrete soluble Sema3A-AP or Sema3C-AP recombinant proteins, respectively.

First, 100 µl of each sample medium was mixed with 100 µl alkaline phosphatase substrate solution (Sigma-Aldrich). After incubation at 37 °C for 3 and 48 h, the amount of converted product, indicated by the colorimetric reaction, was quantitated by determining the optical density (OD, A405 nm) with a 96 well micro plate reader (Mithras LB940, Berthold Technologies). Every sample per condition was assayed in duplicate (double determination). After measuring the OD at 405 nm, the averages were calculated and the background subtracted.

2.3.3 Confirmation of the conditioned media by western blot analysis

Control, Sema3A-AP or Sema3C-AP conditioned media were concentrated and harvested as described in Section 2.2.4.5 being stored at -20 °C until the moment of use. An aliquot of each media was thawed on ice and prepared (mixing 10 µl of sample; 5.6 µl water; 2.4 µl reducing agent (10 X); 6 µl sample buffer (4 X); for a total of 24 µl per load). The samples were separated by 12 % acrylamide SDS-PAGE (NuPAGE) and electrotransferred onto a nitrocellulose membrane. Membranes were blocked in blocking solution for 30 to 60 min and then incubated overnight, at 4 °C, with a specific primary antibody against Sema3A or Sema3C (1:1000, R&D). After washing in TBS-T 4 times, 8 min each, the membrane was incubated 1 h at room temperature with biotinylated secondary antibodies (1:1000, VECTOR) and washed again in TBS-T 4 times, 8 min each. The membrane bound proteins were visualized with immunoperoxidase detection reagent (Vectastain ABC kit) under exclusion from light.

2.4 *in vitro* assays

2.4.1 Experimental procedures

2.4.1.1 Collapse Assay

To study the response from the MGE cells to the Sema3A-AP or Sema3C-AP conditioned media, 100µl of MGE single cells (80.000 cells/ 100µl culture media, Section 2.2.3.3) were

plated onto glass coverslips. The glass coverslips were precoated with a mixture of 19.5°µg/ml laminin and 5 µg/ml poly-L-lysine (PLL) in GBSS for 30 min. After 2 h attachment in FBS-containing single cell medium, the medium was replaced for neurobasal FBS-free culture medium and incubated for 46 h. Next, MGE cells were incubated for 2 h with control, Sema3A-AP or Sema3C-AP conditioned media diluted to a tenfold concentration (described in Section 2.2.4.4) in freshly warmed neurobasal FBS-free culture medium (37 °C). After 2 h exposure the dissociated cells were fixed for 15 min with 4 % PFA/PBS and further immunostaining were performed.

2.4.1.2 Co-localization of Sema3C-AP recombinant proteins and Nrp1 receptors in MGE derived cells

The co-localization of Sema3C-AP recombinant protein to MGE single cells was tested. As described in "2.2.6.1 Collapse assay", dissociated MGE cells were prepared, 100 µl were plated onto laminin/PLL coated glass coverslips with a density of 80.000 cells/100 µl culture media, (Section 2.2.3.3) and cultured in warm FBS-containing cultured medium. After cells attached to the coverslips for 2 h, the culture media was replaced for neurobasal serum-free culture medium (37 °C). Cells were incubated for a total of 46 h and exposed for 2 h to control, Sema3A-AP or Sema3C-AP tenfold conditioned media diluted in freshly warmed FBS-free neurobasal medium (37 °C). After 2 h exposure, the dissociated cells were fixed for 15 min with 4 % PFA/PBS and further immunostaining were performed.

2.4.1.3 Stripe assay

The stripe assay allows the use of two substrates in alternate stripes, to test the ability of axons or dissociated migratory cells to distinguish between the presented substrates (Vielmetter, Stolze et al. 1990, Knoll, Weinl et al. 2007, Rudolph, Zimmer et al. 2010, Zimmer, Schanuel et al. 2010). During culture, axons or migratory cells grow more on the bands with the substances of preference. Results where more axons or cells lay on the tested protein stripes indicate attraction, whereas more axons or cells on the control stripes indicate repulsion to the tested protein.

For the stripe formation, silicone matrices were used. The silicon matrices were created by the Max-Planck Institute for Developmental Biology (Tübingen, Germany). HNO_3 treated glass coverslips were placed on the silicone matrices and 25 µl of a 50 µg/ml human

Semaphorin 3C-Fc solution (Sigma-Aldrich) in PBS were injected into the matrix channels. After incubation at 37 °C for 2 h, the coverslips were rinsed with PBS and coated with 19.5°µg/ml laminin and 5 µg/ml PLL for 30 min, to obtain alternating stripes of labeled Sema3C-Fc and unlabeled control protein (laminin/PLL).

Dissociated neurons (90.000 cells/100 µl media, Section 2.2.3.3) were added to the cover slips, after 2 h incubation the cells were adhered to the substrate. Thus, 1 ml of warmed (37°C) culture medium was added carefully. After 2 DIV, cells were fixed with 4 % PFA/PBS.

2.4.1.4 Boyden chamber

Boyden chamber assays (Santiago and Erickson 2002, Zimmer, Garcez et al. 2008, Steinecke In submission) were performed to test whether Sema3C-AP conditioned media affect MGE-derived cells migration. For performing the assays haptotaxis kits were used (Cell Biolabs). As detailed in Figure 7 A, this kit includes an insert containing a membrane of 8 µm pores, precoated with collagen. The membrane serves as a barrier and creates an upper and lower compartment that can be filled with two different media, diffusing and creating a gradient. Cells are placed onto the upper compartment (Fig. 7 B) and are able to migrate through the membrane as expected under control conditions (Fig. 7 C). How cells migrate will depend on the effect of the cue they are exposed to. Placed in the lower well of the chambers, attractive cues will increase migration (Fig. 7 D) whereas repellent cues will reduce it.

For performing the experiment, under sterile conditions, a 24 well migration plate (4 °C) was warmed up at RT for minimum 10 min. 500 µl of neurobasal FBS-free culture medium containing tenfold control or Sema3C-AP conditioned media was placed in the lower wells of the Boyden chambers (Fig. 7 B). Avoiding any bubble was assured that the membranes were in contact with the lower media. Next, each inserts were filled up with 300 µl cell suspension (90.000 cells/100 µl media, Section 2.2.3.3) and incubated for 6 h (Fig. 7 B).

After the incubation time, the medium was removed from the chambers. Next, the cells in the net were fixed 15 min with 4 % PFA/PBS and the nuclei stained with 0.04 µg/ml DAPI. Afterwards, the top of the membranes were gently wiped with cotton swabs removing the non-migratory cells. Finally, the membranes were cut out with a scalpel, placed upside down on a slide microscope and embed with Mowiol.

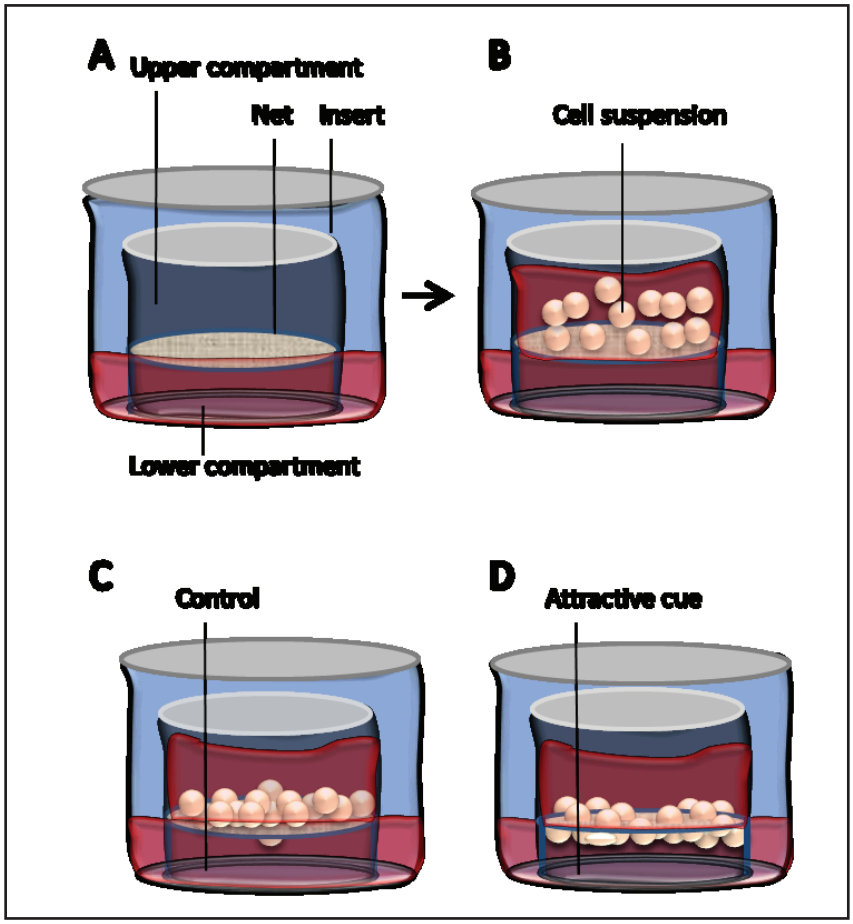


Figure 7. Boyden Chamber assay. (A) Scheme of the parts of a Boyden chamber. (B) Cell suspension is placed in the upper compartment of the insert and FBS-free cultured media with the cue tested in the lower compartment. (C-D) After 6 h incubation in the different media, the cells will migrate. (D) More migration through the net shows attraction to the tested cue. FBS, Fetal bovine serum.

2.4.1.5 MGE explants cocultured with cell aggregates producing Sema3A-AP, Sema3C-AP or control recombinant proteins

Coculture assays (Ruediger, Zimmer et al. 2013, Steinecke In submission) were performed to analyze the migration of MGE neurons in a three dimensional matrix under the influence of Sema3A-AP or Sema3C-AP gradients, created using Sema3A-AP or Sema3C-AP HEK cell aggregates in plasmaclots of chicken plasma crosslinked with thrombin.

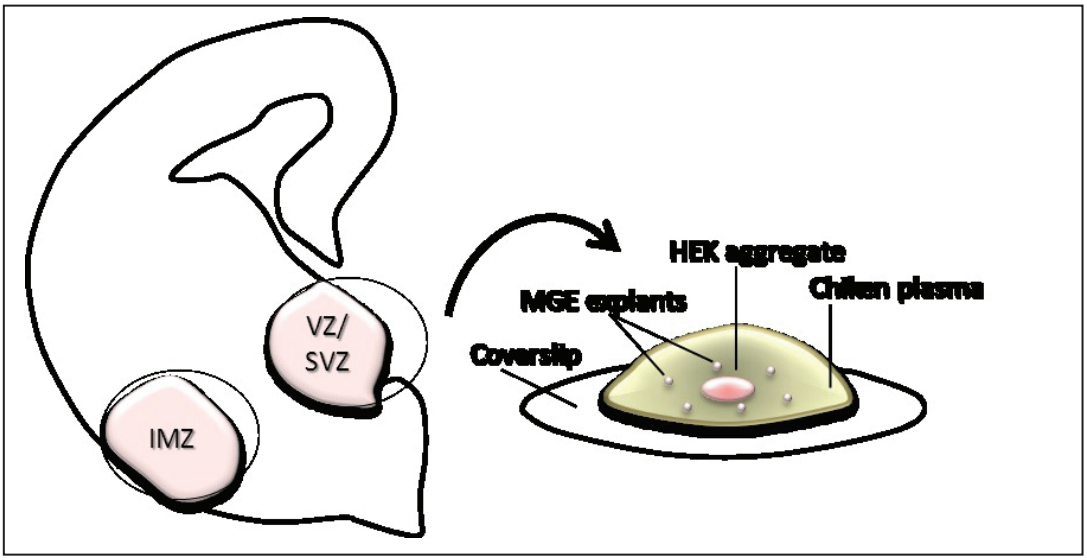


Figure 8. Experimental design of Coculture assays. VZ/SVZ or IMZ from the MGE explants (E14.5) were placed around aggregates of untransfected (control) or secreting Sema3A-AP or Sema3C-AP HEK cells, in chicken plasma, cross-linked with thrombin. VZ/SVZ, Ventricular/subventricular zone; IMZ, Intermediate zone; MGE, medial ganglionic eminences; E Embryonic day; HEK, Human embryonic kidney cells . Modified from (Lehmann, Steinecke et al. 2012).

As illustrated in Figure 8, to prepare cocultures, a HEK cell aggregate (Section 2.2.4.5) was placed in the middle of a drop of 20 µl chicken plasma (Sigma-Aldrich) on non-coated cover slips (washed with HNO3), using a spatula. Afterwards, 5 to 7 MGE explants from the VZ/SVZ or IMZ, were placed at 1°mm from the HEK aggregate and at 1 mm from the next explant. Then, 10 µl of fresh GBSS/thrombin mixture was carefully added, mixing constantly. If necessary, the positions of the explants were corrected. The plasma coagulated during 15 to 30 min at room temperature. Subsequently, 2 ml methylcellulose medium were added. After 2 days in vitro, the cocultures were fixed for 30 min with 4 % PFA/PBS and embedded in Mowiol. This procedure was performed with untransfected, Sema3A-AP or Sema3C-AP HEK cells aggregates.

2.4.2 Immunohistochemistry

Immunostaining was performed on MGE single cells after 15 min fixation with 4 % PFA/PBS at RT. After removal of the fixation solution, the coverslips were washed 3 times for 8 min in PBS containing 0.1 % Triton-X to permeabilize the cells. The cells were incubated for 30 min in blocking solution with the appropriated serum (goat or donkey depending on the secondary antibody). After overnight incubation with the primary antibody in a humid

chamber at 4 °C, another washing cycle was performed, 3 times, 8 min each, and the cells were incubated 1 h with the secondary antibody, in a humid chamber, at RT and covered from light. After the last cycle of washing: 3 times, 10 min each, one washing step was performed with 0,04 µg/ml DAPI (Sigma) in PBS. The coverslips were rinse in distillated water, mounted on slides, embedded with Mowiol and stored at 4°C for further analysis.

Primary and secondary antibodies are detailed in the Chapter 8, Section 8.2 Reagents. For double immunostaining, the two primary or secondary antibodies were supplied as a mixture. Controls without using primary antibodies resulted only in back ground signals.

Collapse assay: the F-actin filaments of the cells were marked using phalloidin conjugated to Rhodamine (1:100, Biotium), making possible to detail the morphology of neuronal processes.

Co-localization: after fixation, one additional washing step was performed with warm PBS (65 °C) to eliminate any intrinsic alkaline phosphatase present in the cells. Immunostaining was performed using antibodies against Neuropilin 1 (1:1000, R&D) and Placental alkaline phosphatase (PLAP) (1:100 ABD-serotec).

Stripe assay: in order to visualize the Human Sema3C- Fc recombinant chimera, bound to the stripes, anti-human conjugated to Alexa 488 (30 µg/ml, Invitrogen),was applied after fixation, following the same procedure as with a secondary antibody.

2.4.3 Microscopy

Collapse assay: oil 100 X objective (Zeiss Achroplan, numerical aperture (NA) 1.25) was used with fluorescence excitation to visualize the phalloidin fluorescence signal after immunostaining. The excitations of the neurons stained with phalloidin were performed with the light wavelength 530-560 nm. MGE single cells were photographed using a digital camera (Spot, Diagnostic instruments) in combination with the Spot software and a Zeiss Axioverts S100 inverted fluorescence microscope (Zeiss, Germany).

Co-localization assay: water 63 X objective was used to scan with a confocal laser scanning TCS SP5 (Leica) and the software Leica Application Suite Advanced Fluorescent lite (LAS Af lite; Leica 2011)

Stripe assay: 20 X objective (Zeiss Plan Neofluar, NA 0.5) was used in combination with fluorescence excitation to visualize the stripes. MGE single cells on the stripes were

photographed using a digital camera (Spot, Diagnostic instruments) in combination with the Spot software and a Zeiss Axioverts S100 inverted fluorescence microscope (Zeiss, Germany).

Boyden chamber assay: MGE single cells were visualized using a Zeiss Axioverts S100 inverted fluorescence microscope (Zeiss, Germany).

Coculture assay: phase contrast pictures were taken using a 5 X objective (Zeiss Plan Neofluar, NA 0.15) for the location of the explants in relation with the HEK cell aggregate, or 10 X objective (Zeiss Plan Neofluar, NA 0.3) for making a closer picture to each explant analyzed. Explants were photographed using a digital camera (Spot, Diagnostic instruments) in combination with the Spot software and a Zeiss Axioverts S100 inverted fluorescence microscope (Zeiss, Germany).

2.4.4 Analysis of *in vitro* assays

2.4.4.1 Collapse assay.

Pictures were taken using the microscope described in Section 2.2.8. The responses from the growth cones and processes were quantified blindly for every condition using the ImageJ software. The growth cone response was analyzed by measuring its area (Fig. 9 A) and measuring its length and width (Fig. 9 B).

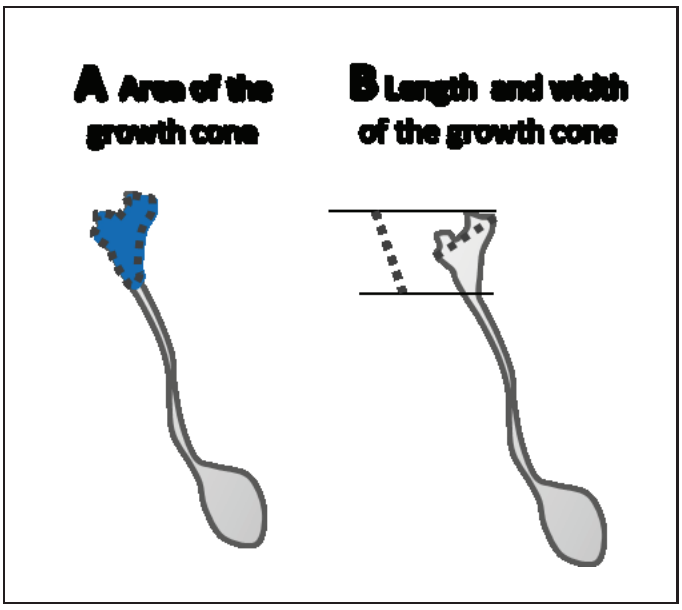


Figure 9. Guidelines for collapse assay data processing. (A-B) Growth cone responses to Control, Sema3A-AP or Sema3C-AP conditioned media was analyzed measuring with ImageJ: (A) area of the growth cone shown in blue, obtained marking its perimeter (dash line). (B) Length of the growth cone, taken from its initial segment to the end of the growth cone and width obtained on the broader part of its border. Dashed black lines show the outline used for quantification.

2.4.4.2 Co-localization assay.

Cells were scanned and the co-localization was outline in an X and Y line scan through a single optical plane using Leica Application Suite Advanced Fluorescent lite program (LAS Af lite; Leica 2011).

2.4.4.3 Stripe Assay.

Pictures were taken and the fluorescent and phase contrast photo-micrographs were overlay to visualize the Sema3C-Fc/Alexa 488 stripes (Section 2.4.3). At least 7 random frames were pictured per coverslip. The distribution of the neurons (location of the cell body) on the different types of stripes was counted, using the cell counter plugin of ImageJ (Rudolph, Zimmer et al. 2010, Zimmer, Schanuel et al. 2010). Total number of neurons on the alternating stripes was corrected according to the varying widths of the stripes. The percentage of cells on the respective stripes was calculated for each frame, results were presented as percentage.

2.4.4.4 Boyden chamber assay.

The migration of the cells was quantified using a fluorescence inverted microscope (Axioverts S100, Zeiss) with 20 X objective (Zeiss) and fluorescent excitation to visualize the DAPI stained nuclei (Section 2.2.8). Per membrane, at least 15 frames were analyzed. Three chambers/inserts were used per condition for each experiment. The number of cells that migrated through the membrane in control conditions was set to “1” and the cells that migrated into the lower compartment under sema3C-AP were calculated relatively. As described in: (Steinecke In submission).

2.4.4.5 Coculture assay

2.4.4.5.1 Guidance index.

To quantify the migration of MGE neurons cocultured with control, Sema3A-AP or Sema3C-AP gradients, only explants that outgrowth in all their surfaces were taken into account. Phase contrast pictures were taken as described in Section 2.2.8. Next, the growth of the MGE explant analyzed was scored as described by Marin et al., (2003). “0” was given if the explant had equally outgrowth (Fig. 10 B); -1 or -2 for moderate or strong repulsion, when cells are migrating away from the explant (Fig. 10 C) and +1 or +2 for moderate or strong attraction when cells are migrating towards the explant (Fig. 10 A). A “guidance index” was calculated from the averages of all scores from control, Sema3A-AP or Sema3C-AP cocultures. A guidance index with a positive value implies attraction whereas a negative value represents repulsion.

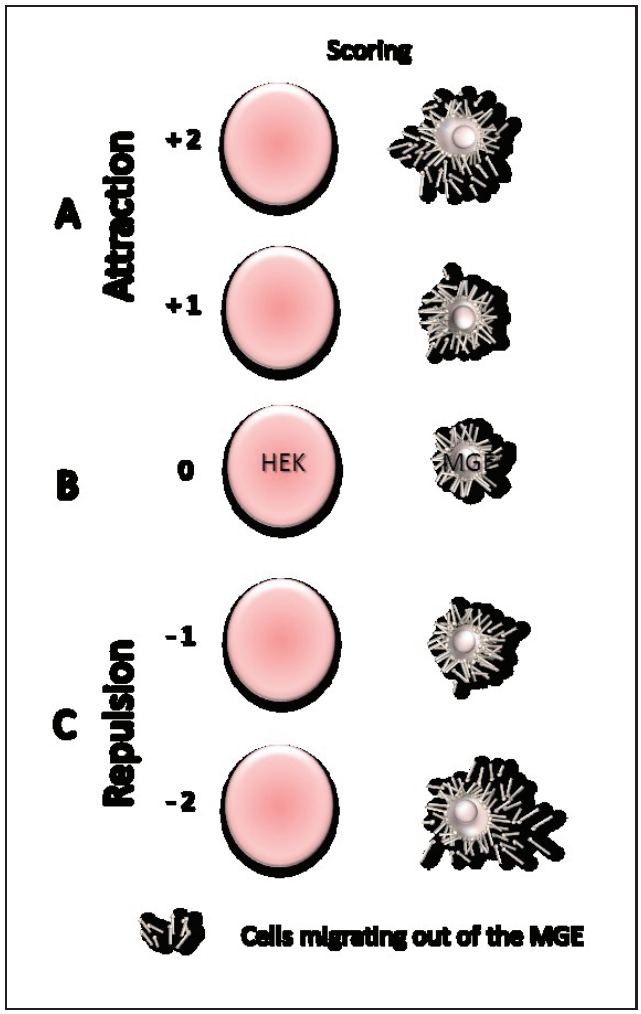


Figure 10. Semiquantification of coculture assays. Scheme of MGE explants, co-cultured 2 days in vitro with aggregates of HEK cells, with its initial shapes and outgrow. (A, C) Unequal outgrowth refers to: (A) More cells migrating towards the aggregate, when exposed to an attractive cue (C) Less cells migrating towards the aggregate, when exposed to a repellent cue. (B) Representation of equally outgrowth is expected under control conditions or when exposed to a neutral cue. MGE, Medial ganglionic eminences. Modified from (Marin 2003).

2.4.4.5.2 Coculture assay, distance migrated by derived neurons.

The distance migrated by MGE-derived neurons form VZ/SVZ explants, was measured using the ImageJ program. For this analysis, micrographs of each explant were taken (Section 2.4.3) and prepared. The initial shape of the explant was divided in six parts (one virtual division was parallel to the border of the HEK aggregate). As illustrated in Figure 11, with these divisions, six sectors were obtained, Sector 2 being the "proximal" and Sector 5 the "distal" area, in relation to the position of the HEK cell aggregate (Bagnard, Lohrum et al. 1998, Marin 2003, Flames, Long et al. 2004, Zylbersztejn, Petkovic et al. 2012).

In each proximal and distal area, the 10 farthest neurons that migrated out of the explant were measure using ImageJ, by making a line between the initial border of the MGE explant and the soma of the cell.

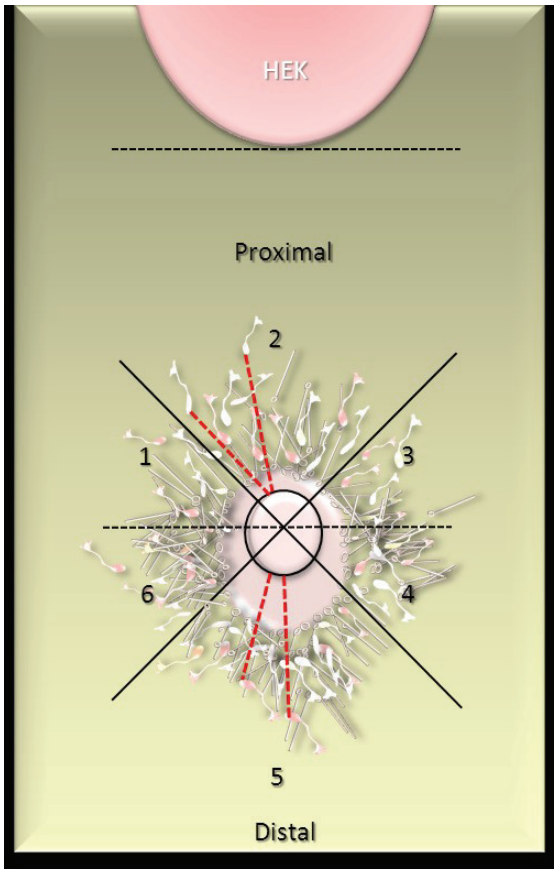


Figure 11. Measurements of the distance migrated by MGE-derived neurons in coculture assays. Using micrographs, the initial shape of the MGE is marked with a black line. Six sectors were created in the MGE explant, setting a virtual line parallel to the border of the HEK aggregate (black dashed line). Proximal and distal areas were set perpendicular to the HEK cell aggregate. Dashed red lines illustrate the migration distance of MGE-derived interneurons, measured from the initial border of the MGE explant to the soma. MGE, Medial ganglionic eminences.

2.4.4.5.3 Length of the processes of MGE neurons cocultured with cell aggregates producing Sema3A-AP, Sema3C-AP or control.

For this quantitative analysis, the coculture micrographs with virtual divisions were used, as described in Section 2.4.4.5.2, Figure 11. The neurite lengths of 10 single cells that migrated out of the explant were measured using ImageJ. As shown in Figure 12, the distance from the initial segment of the process to the end of its tip was quantified.

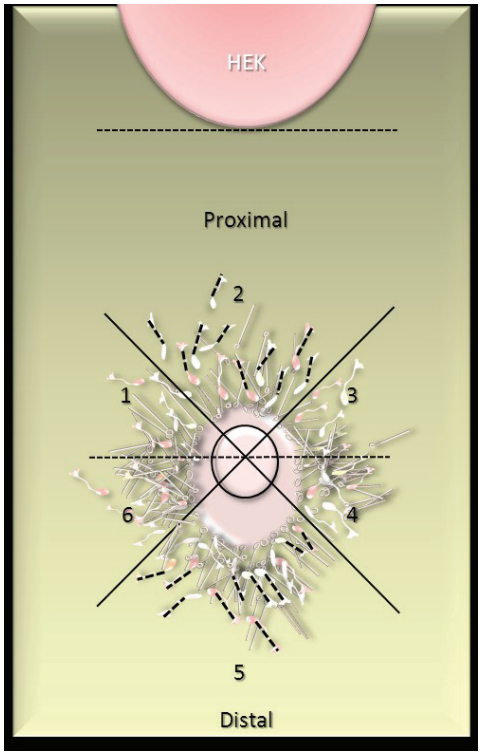


Figure 12. Measurement of the neurite length of MGE-derived neurons in coculture assays. Using micrographs, the initial shape of the MGE is marked with a black line. Six sectors were created in the MGE explant, setting a virtual line parallel to the border of the HEK aggregate (black dashed line). Proximal and distal areas were set perpendicular to the HEK cell aggregate. Dashed lines represent the length of the neuronal processes, outlined for the measurement using the ImageJ program. MGE, Medial ganglionic eminences.

2.4.5 Statistics

Statistical differences were mostly determined with a two-tailed student's t test.

Collapse assay: the results are presented as mean + standard error of the mean (SEM). The Bonferroni correction was applied to the two-tailed student's t test, since is a multiple comparison. Results for each experimental condition are from two independent preparations, "n" refers to the number of analyzed neurons.

Stripes assay: a paired student´s t-test was used, three independent preparations were performed; "n" refers to the number of analyzed pictures. Values are presented as percentage with their mean + standard deviation (SD). Results are from three independent preparations.

Boyden chamber assay: the ciphers are presented as mean + SEM. Results from each experimental condition are from two independent preparations, "n" refers to the number of analyzed frames.

Coculture assays: for the guidance index, results are presented as mean + SEM; "n" number refers to the amount of explants analyzed. For the distance migrated and process length values are presented as mean + SEM; the "n" number refers to the amount of neurons analyzed. Results for each experimental condition are from at least three independent preparations.

Chapter 3: Results

Cortical interneurons derive from the medial ganglionic eminence (MGE), the caudal ganglionic eminence (CGE) and the preoptic area (POA) of the basal telencephalon (Nery, Fishell et al. 2002, Wonders, Taylor et al. 2008, Batista-Brito, Rossignol et al. 2009, Gelman, Griveau et al. 2011). During development they migrate tangentially into the embryonic neocortex, following spatial and temporal distinct routes in response to a combination of chemoattractive and chemorepulsive cues towards their cortical destination (Zimmer, Schanuel et al. 2010, Zimmer, Rudolph et al. 2011, Ma, Wang et al. 2013, Marin 2013, McKinsey, Lindtner et al. 2013, Molnar and Butt 2013, van den Berghe, Stappers et al. 2013, Guo and Anton 2014). Studies have suggested that Semaphorin proteins like Semaphorin 3A (Sema3A) repelled cortical interneurons, avoiding their innervation into nontarget regions like the striatum (Marin, Yaron et al. 2001, Hernández-Miranda et al. 2011, Zimmer, Schanuel et al. 2010).

The expression patter of another Semaphorin III protein: Semaphorin 3C (Sema3C) and its possible binding partners Neuropilin 1 (Nrp1), Neuropilin 2 (Nrp2) and Plexins (Marin, Yaron et al. 2001, Hernández-Miranda et al. 2011, Zimmer, Schanuel et al. 2010, Castellani, Falk et al. 2004, Ruedinger, Zimmer et al. 2013), is consistent with a model in which Nrp1 expressing interneurons derived mainly from the MGE, and Nrp2- expressing interneurons derived mainly from the POA, might follow gradients of Sema3C towards the neocortex. Once the neocortex is reached, Nrp1 or Nrp2 expressing cortical interneurons might use Sema3C-expressing cells as a substrate en route to its target areas. However, Sema3C possible functions on guiding migrating cortical interneuron have not been examined. Therefore, in this study, the potential role of Sema3C on the tangential migration of cortical interneurons derived from the MGE was investigated. Furthermore, the repellent effect described for Sema3A on migrating cortical interneuron, was studied in more detail.

3.1 Confirmation of the recombinant proteins Sema3A-AP or Sema3C-AP expressed by the stable cell lines.

In most of the in vitro assays performed in this study, recombinant proteins produced by stable HEK cell lines were used. Two stable HEK cell lines, one producing Sema3C-AP and the other Sema3A-AP, secreted the recombinant proteins directly into the culture media. Both

recombinant proteins produced by HEK cell lines, Sema3A or Sema3C, are fused to an alkaline phosphatase tag (-AP) (Bagnard, Lohrum et al, 1998). Thus, the recombinant proteins, secreted by the stable HEK cell lines, are named Sema3A-AP and Sema3C-AP.

3.1.1 Detection of the recombinant Sema3A-AP or Sema3C-AP proteins via western blot

In order to confirm the expression of the recombinant proteins Sema3A-AP or Sema3C-AP through time, the HEK cells were incubated for 5, 8 or 24 h in 6 ml fresh medium. The expression of the Semaphorin-AP proteins were equally examined in HEK cell aggregates (section 2.2.4.5), by cultured them for 12 h in 20 µl fresh culture medium. Each cell line was expected to secrete soluble Sema3A-AP or Sema3C-AP directly into the culture media. Untransfected HEK cells were used as negative control, and expected to secrete only intrinsic proteins into their culture medium.

The medium of the HEK cells and aggregates were harvested and used for sample preparation and western blot analysis, being subjected to SDS-PAGE, electrotransferred onto a PVDF membrane and immunostained with specific antibodies against Sema3A or Sema3C as detailed in Section 2.4.2. Inactive Sema3A is predicted to have a band size of 120 kDa and activated Sema3A 95 kDa or 62 kDa, depending on the processed variant of the protein (Adams Lohrum et al., 1997). For activated Sema3C, a size of 130 kDa or 95 kDa has been reported. Because the recombinant proteins are fused to an alkaline phosphatase with a presumed size of 55 kDa (Püschel et al., 1995), the expected bands were 175 kDa for inactive Sema3A-AP and 150 kDa or 117 kDa for the splicing variants of active Sema3A-AP. For activated Sema3C-AP the expected sizes were 185 or 150 kDa.

Bands in the western blot had the predicted sizes (Fig. 13) confirming the presence of Sema3A and Sema3C fussed with alkaline phosphatase. Therefore, the secretion of Sema3A-AP and Sema3C-AP by the HEK-cells and aggregates into the culture media was confirmed. The strongest bands represented Sema3A-AP and Sema3C-AP proteins on samples from cell aggregates (Fig. 13 B), ratifying that aggregates can still secrete a considerable amount of tagged proteins. As expected, the secreted proteins accumulated in the culture media, increasing proportionally over the time the cells were cultured (Fig. 13 A).

Even with only 5 h incubation, a band of recombinant either Sema3A-AP or Sema3C-AP protein was detected, the strongest band was obtained with 12 h incubation under cell aggregate conditions. Therefore, when performing in vitro assays like the coculture, were

cell aggregates incubated during 2 DIV in a plasma clot, a strong gradient of the recombinant Semaphorin-AP proteins is likely to occur. Also, for preparing the conditioned media (section 2.2.4.4) 3 days of incubation guarantee enough time for the HEK cells to secrete a high amount of recombinant protein into the culture medium.

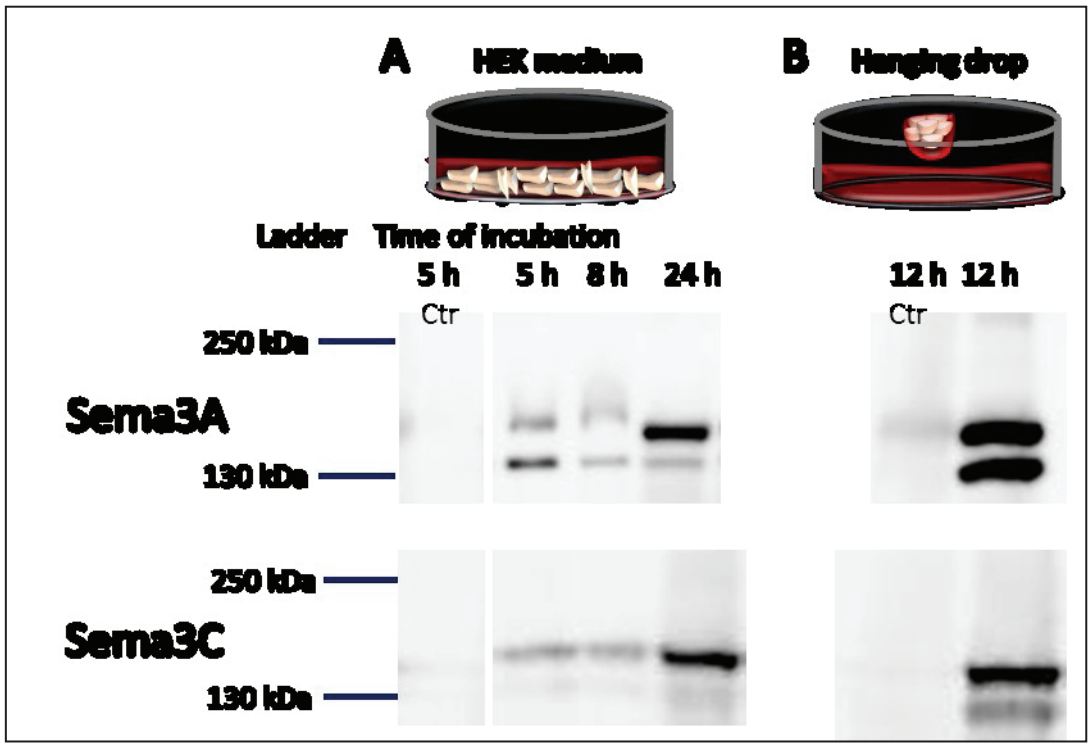


Figure 13. Confirmation of Sema3C-AP and Sema3A-AP secretion through western blot analysis. (A) 6 ml fresh media were applied on 80 % confluent Sema3A-AP and Sema3C-AP stable HEK lines and control untransfected HEK cells. HEK cells secreted directly control, Sema3A-AP or Sema3C-AP recombinant proteins into the 6 ml culture media. The media were collected after 5, 8 and 24 h. (B) 6 ml fresh media were applied on 80 % confluent Sema3A-AP and Sema3C-AP stable HEK lines and untransfected HEK cells used as control. Hanging drops were prepared as detailed in Section 2.2.4.5. Cell aggregates secreted directly control, Sema3A-AP or Sema3C-AP recombinant proteins into the 20 µl culture media and collected after 12 h incubation. (A,°B) 10 µl of each medium was separated on a 12 % SDS gel and blotted on PVDF membrane. The presence of each protein was confirmed using specific antibodies. In all Semaphorin incubation conditions clear bands were visible with an increase in intensity over time. The strongest bands were presented with the hanging drop media samples (B, 12 h incubation). Using a specific antibody against Sema3A the predicted bands, 175 and 150 kDa for activated Sema3A-AP were obtained. Also, using a specific antibody against Sema3C proteins the predicted bands, 185 and 150 kDa for activated Sema3C-AP, were obtained. As expected, no significant bands were obtained under control conditions. Ctr, Control.

3.1.2 Confirmation of the integrity of the recombinant Sema3A-AP or Sema3C-AP proteins, secreted by the respective HEK cell lines.

In order to test the integrity of the alkaline phosphatase tag and indirectly confirm the secretion of Sema3A-AP and Sema3C-AP protein through time by the stable cell lines, a colorimetric assay was performed (Fig. 14). For the colorimetric assay, the two different stable HEK cells lines secreted soluble Sema3C-AP or Sema3A-AP recombinant proteins during 1, 3, 4, 5, and 24 h incubation directly into the culture medium. Untransfected HEK cells were used as negative control, incubated under the same conditions and expected to secret only intrinsic proteins from HEK cells. The AP activity was measured in each culture supernatant using p-nitrophenyl phosphate as a non-biological transparent substrate. The alkaline phosphatase catalysed the conversion of the transparent substrate to yellow p-nitrophenol. The color change is an indicator for the presence and integrity of the enzyme. Therefore, for analyzing the quality of the AP, 100 µl medium was incubated with 100 µll 4-nitrophenyl phosphate for 3 h and 48 h. The amount of converted product was measured by determining the optical density (OD) at 405 nm, using a 96-well plate reader. Every sample per condition was assayed in duplicate (double determination). After measuring the OD at 405 nm, the averages were calculated and the background subtracted.

Accordingly, control media remain unchanged, since no AP tag is present. On contrary, media from Sema3A-AP and Sema3C-AP stable HEK cells lines contained AP tagged proteins, indicated by a measurable colorimetric reaction in a time dependent manner. As shown in Figure 13, the amount of Sema3C-AP protein in the media increased proportionally with the time of incubation, duplicating after 24 h (OD405 = 1.3 after 1 h incubation, OD405 = 1.9 after 5 h incubation, OD405 = 2.8 after 24 h incubation, n = 2 dishes). In contrast, the amount of Sema3A-AP proteins was similar between all the different incubation conditions, around OD405 = 2.6 to 2.8 during the first 1 to 5 hours, reaching OD405 = 2.9 after 24 h incubation (n = 2 dishes). One possible explanation for the measured differences between Sema3A-AP and Sema3C-AP samples (Fig. 14), is that reflects less protein production.

As expected, when the samples were measured after 48 h reaction, the p-nitrophenyl substrate reached saturation, obtaining OD405 values around 2.7 and 2.9 for both Sema3-AP proteins harvested after 1 h to 5 h incubation. A peak was observed after 24 h incubation with an increase of OD405 = 3.2.

Overall, the presented results indicate that the AP-enzyme fussed to Sema3A and Sema3C recombinant proteins can serve as marker to identify the corresponding tagged Semaphorins. Using this Sema3-AP tagged recombinant proteins in additional in vitro assay like co-localization or binding experiments, Sema3A-AP or Sema3C-AP receptors could be identified.

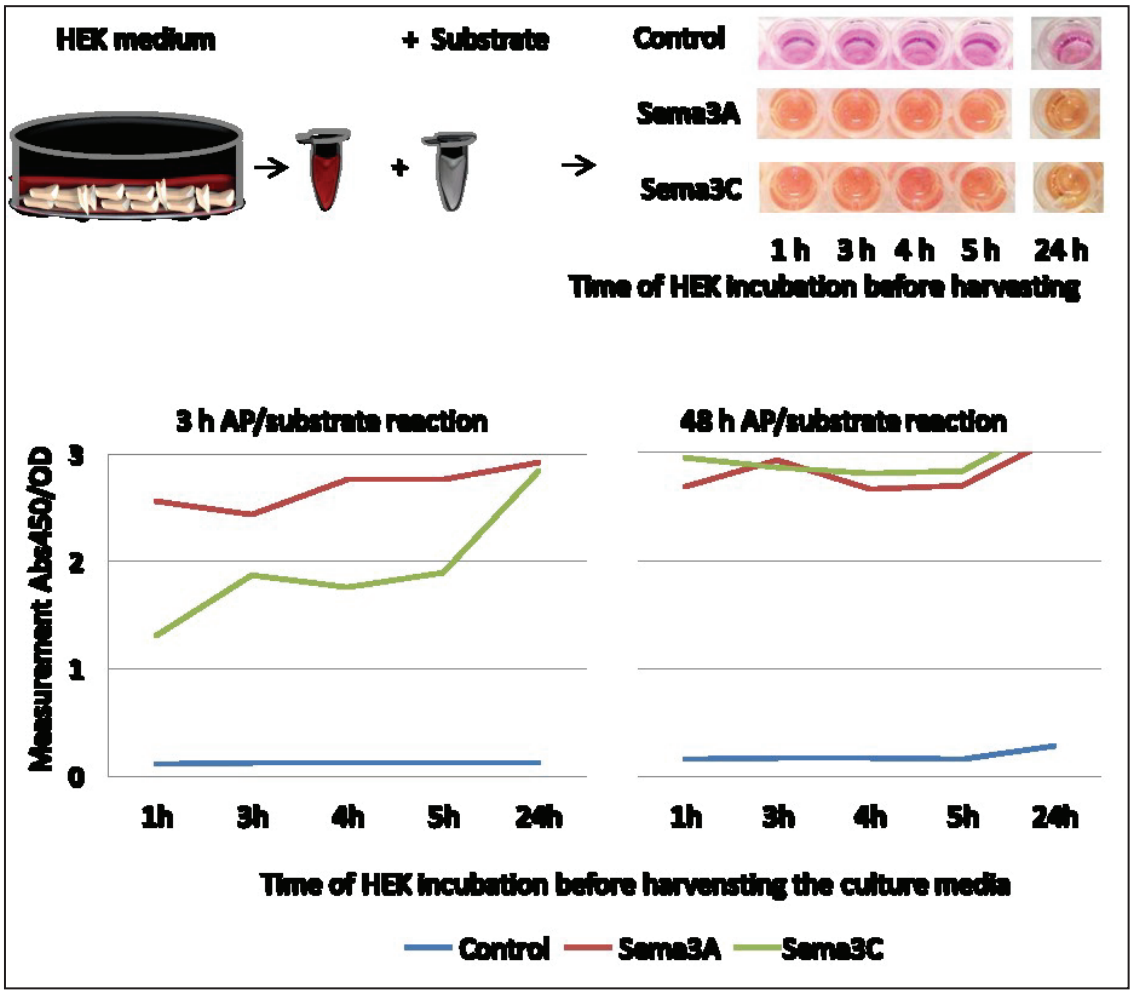


Figure 14. Colorimetric analysis of Sema3A-AP or Sema3C-AP recombinant proteins. To test the functionality and integrity of the AP- tagged Sema3A-AP and Sema3C-AP recombinant proteins, 80 % confluent HEK control, Sema3A-AP and Sema3C-AP secreted directly into 6 ml culture media recombinant proteins. After 1, 3, 4, 5 and 24 h, each medium was collected and 100 µl were transferred to a 96 micro well plate. On each 100 µl sample, 100 µl of 4-nitrophenyl phosphate substrate was added. After 3 and 48 h, OD450 measurements were made. The change of color shows the presence of integral and functional AP tag and therefore the presence of the Semaphorin-AP tag proteins. As expected, only the media from Sema3A-AP and Sema3C-AP reacted. No color reaction was observed in the control samples. AP, Alkaline phosphatase.

3.1.3 Detection of the recombinant Sema3A-AP or Sema3C-AP proteins on the concentrated conditioned media via western blot

In the case of the conditioned media, the presence of Sema3A-AP or Sema3C-AP was confirmed through western blot. Such confirmation was performed with samples from before and after the collection and concentration of any molecule bigger than 100 kDa and therefore Semaphorin-AP molecules, using a filter device (Section 2.2.4.4). Aliquots from control, Sema3A-AP and Sema3C-AP conditioned media were used for sample preparation and western blot analysis, being subjected to SDS-PAGE, electrotransferred onto a nitrocellulose membrane and immunostained with antibodies against Sema3A or Sema3C as detailed in Section 2.4.2.

Bands of predicted sizes were observed (Fig. 15) confirming the concentration of Sema3A-AP and Sema3C-AP on the conditioned media. As described before, the expected bands were 175 kDa for inactive Sema3A-AP and 150 kDa for the splicing variant of active Sema3A-AP. For activated Sema3C-AP the expected band sizes were 185 kDa or 150 kDa. (Adams, Lohrum et al. 1997, Püschel et al., 1995).

In conclusion, western blot analyses confirmed that Sema3A-AP or Sema3C-AP recombinant proteins are concentrated in the conditioned media. This validates the conditioned media for further use in the in vitro assays.

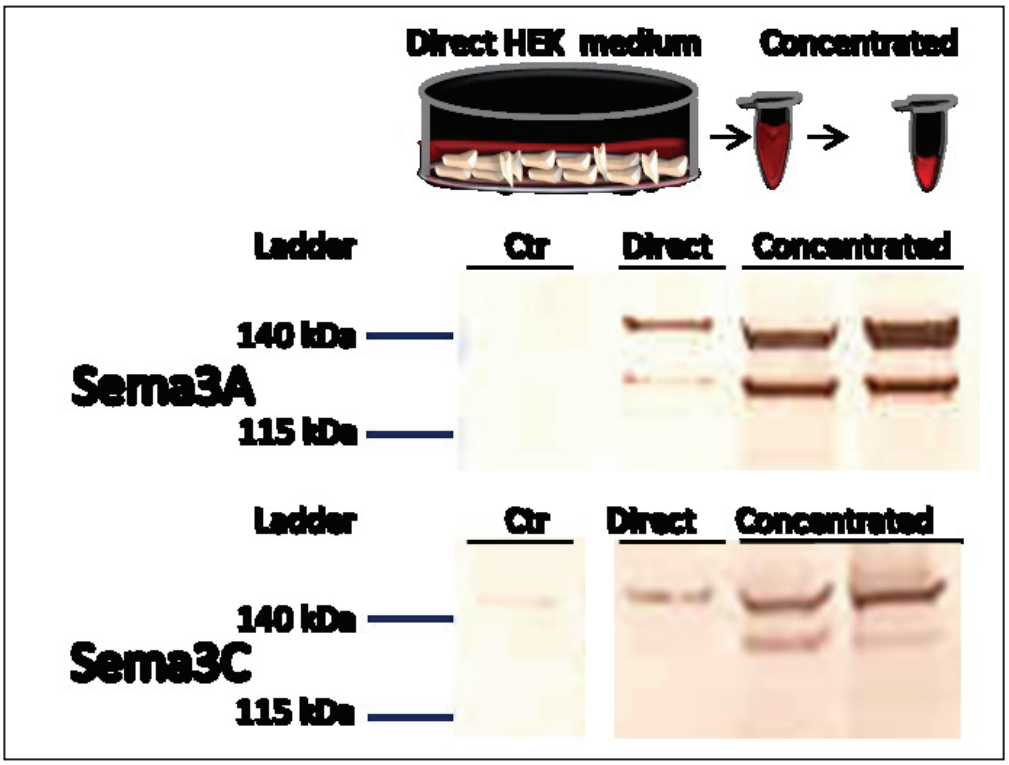


Figure 15. Presence of concentrated recombinant protein Sema3A-AP or Sema3C-AP in the conditioned media, confirmed by western blot analysis. 6 ml fresh media were applied on 60 % confluent Sema3A-AP and Sema3C-AP stable HEK lines and control untransfected HEK cells. HEK cells secreted directly control, Sema3A-AP or Sema3C-AP recombinant proteins into the 6 ml serum free culture media and were collected after 3 days incubation and named "Direct media". Using a filter device, molecules bigger than 100 kDa from each direct media were collected and concentrated creating the so call "conditioned media". 10 µl of each direct and concentrated medium was separated on a 12 % Bis/tris-SDS gel and blotted on nitrocellulose membrane. The presence of each protein was confirmed using specific antibodies. In all Semaphorin media, clear bands were visible with an increase in intensity on the concentrated media. Using a specific antibody against Sema3A, the predicted bands around 150 kDa for activated Sema3A-AP, were obtained. Also, using a specific antibody against Sema3C, the predicted bands, around 150 kDa for activated Sema3C-AP, were obtained. As expected, no significant bands were obtained under control conditions. Ctr, Control.

3.1.4 Recombinant Sema3C-AP proteins were able to bind to the Nrp1 receptor

After showing that Sema3A-AP or Sema3C-AP were secreted by the stable cell lines, that the conditioned media contained concentrated amounts of the proteins of interest, and that the fused AP-tag can serve as a marker, it was important to examine if the artificial recombinant protein Sema3C-AP tagged with alkaline phosphatase was able to bind properly to any interaction partner and to receptors on MGE-derived neurons.

Neuropilin 1 (Nrp1) is present on cortical interneurons derived from the MGE, and is a well-known binding partner for class III Semaphorin protein family, including Sema3C, Sema3A and Sema3F (Castellani and Rougon 2002, Marin, Yaron et al. 2001, Ruediger, Zimmer et al.

2013,Zimmer, Schanuel et al. 2010). Sema3A and Sema3F interactions with Nrp1 have been shown to mediate the guidance of cortical interneurons en route to the cortex (Marin, Yaron et al. 2001, Hernández-Miranda et al. 2011, Zimmer, Schanuel et al. 2010), Therefore, Nrp1 was the perfect candidate for testing whether Sema3C–AP proteins binds to receptors on MGE-derived neurons.

Since E14.5 is the highest period of interneuron migration, all in vitro experiments were done at this developmental stage. Therefore, neurons derived of E14.5 medial ganglionic eminences were cultured for 46 h, and then stimulated 2 h with Sema3C-AP or control conditioned medium (Section 2.2.4.4). Subsequently, double immune staining was performed applying antibodies against Neuropilin 1 and alkaline phosphatase (AP). As illustrated in Figure 16, The Nrp1 was detected on the cultured cortical interneurons (Fig. 16,°red). The stained AP-tag (Fig. 16, green) co-localized with the Nrp1-signal, as illustrated by X and Y line scan through a single optical plane (orthographic representation). These data indicate that the artificial recombinant Semaphorin fused to AP co-localized with appropriate interaction partners in vitro. Thus, the recombinant proteins produced by the stable HEK cell lines can be used for further in vitro experiments.

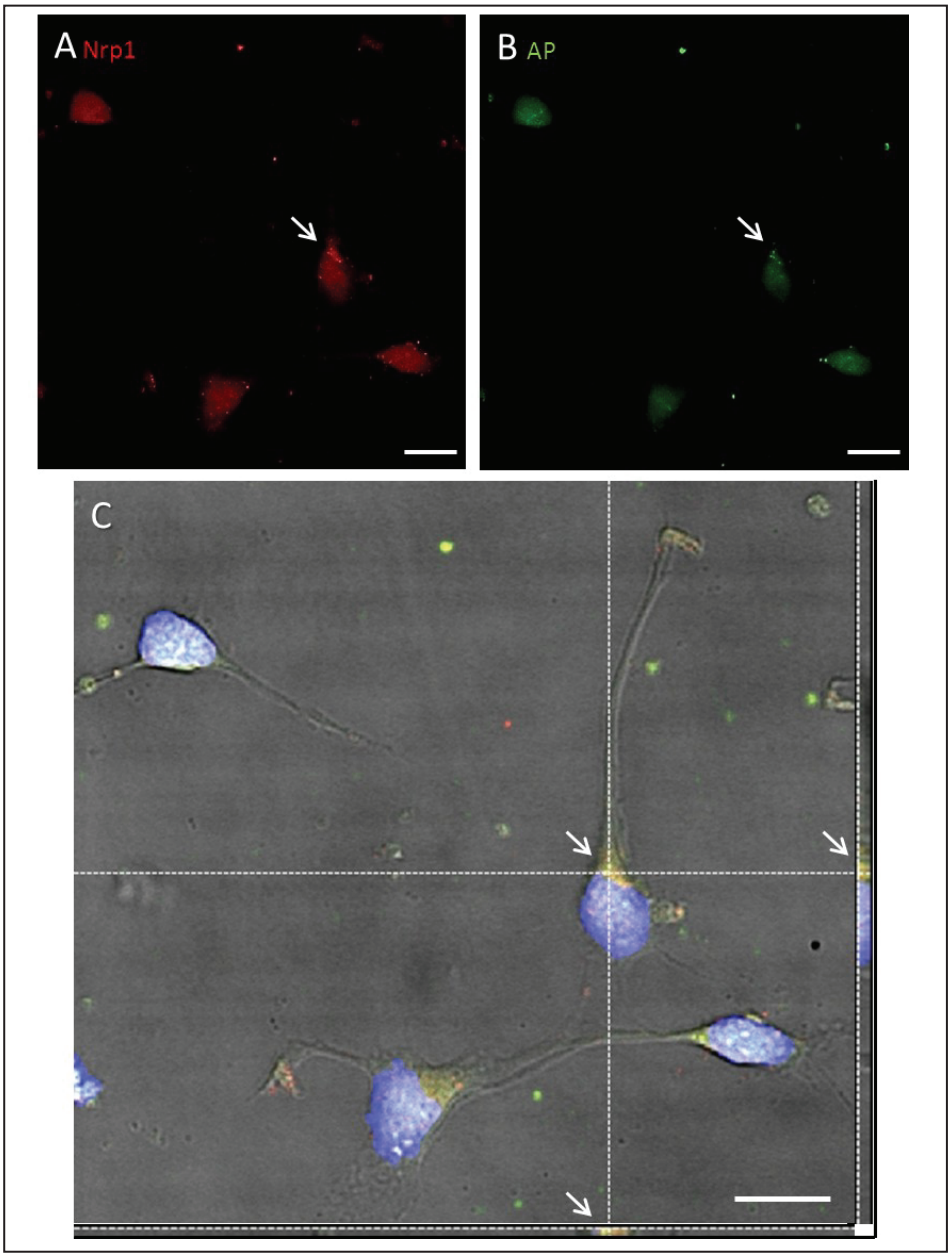


Figure 16. Sema3C-AP recombinant protein co-localized with Nrp1 receptors in MGE single cells. E14.5 single neurons from the MGE were stimulated 2 h with Sema3C-AP or control conditioned medium. After fixation, double immunostaining was performed against (A) Nrp1 in red and (B) AP in green. (C) Co-localization of AP tag fused to Sema3C and Nrp1 is illustrated in an X and Y line scan, through a single optical plane. White arrows highlight AP/Nrp1 co-localization. Two independent experiments were performed. Scale bars equals 10 µm. E14.5, Embryonic day 14; Nrp1, Neuropilin 1; AP, Alkaline phosphatase.

3.2 Functional studies of the role of Sema3C on cortical interneuron migration in vitro

3.2.1 Sema3C is a permissive signal molecule for neurons derived from medial ganglionic eminences (MGEs).

For testing substrate preferences of growing axons or migratory cells, the stripe assay has been widely used (Vielmetter, Stolze et al. 1990, Knoll, Weinl et al. 2007, Rudolph, Zimmer et al. 2010, Zimmer, Schanuel et al. 2010). In such assays, lanes of signaling molecules are created, and the entire platting surface is covered with permissive growth substrates like laminin, poly-L-lysine, and collagen, among others. When culturing neuronal cells on this stripe field, axons or migratory cells move all over. After approximately 2 DIV culturing, results were more axons or cells lay on the stripes with the tested protein indicates an attractive effect, whereas more axons or cells on the control stripes indicate a repulsive protein effect on the neuronal cells.

Previous Sema3A-Fc stripe assays showed that about 60 % of MGE-derived interneurons were located on the control stripes, indicating that Sema3A-Fc is a repellent cue for single MGE-derived neurons (Zimmer, Schanuel et al. 2010). However, no information is available regarding Sema3C effects on neurons derived from the MGE. Thus, to examine if MGE-derived neurons prefer Sema3C substrates, stripe assays were performed. For this reason, alternating stripes of 50 µg/ml recombinant Sema3C-Fc proteins were formed on glass coverslips and the entire surface was covered with a growth-permissive substrate (laminin/poly-L-lysine). Dissociated cells from E14.5 MGEs were prepared (Section 2.2.2.3), plated on the stripes, and cultured for 2 DIV. After fixation, the Sema3C-Fc stripes were visualized with an Alexa488-labeled antibody and the distribution of neuronal cells was quantified.

After analyzing the distributions of the MGE-derived cells on the alternating stripes, 47 ± 1.10 % of the cells were located on the control stripes, while 53 ± 1.10 % were counted on the Sema3C-Fc stripes (Fig. 17; n = 96 frames analyzed; paired Student´s t-test; ** $p \leq 0.01$. Results are from three independent experiments). Thus, MGE-derived cells seem to prefer by a Sema3C-Fc substrate. The quantification shows 6 % more cells on the Sema3C-Fc stripes, indicating a significant attractive effect on MGE-derived interneurons.

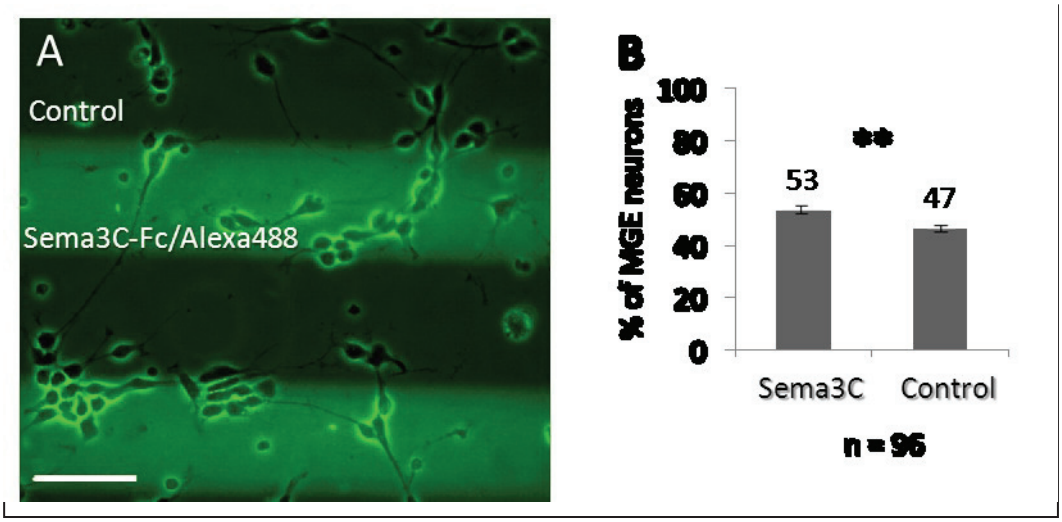


Figure 17. Sema3C-Fc has an attractive effect on cortical interneurons. Glass slides were coated with alternating stripes of 50 µg/ml Sema3C-Fc recombinant protein and entirely covered with a growth substrate (Laminin//PLL). Dissociated E14.5 MGE cells were plated on top of the stripes and their distribution was studied 2 DIV later. (A) Overlay of fluorescent and phase contrast photo-micrographs. (B) Quantification of the distribution of MGE-derived cells growing on alternating stripes of labeled Sema3C-Fc and control. Cortical interneurons show a preference to grow on Sema3C-Fc stripes. Scale bar: 50 µm; n, number of pictures analyzed; Student´s paired t-test: $^{**}p < 0.01$; error bars are SD. Results are from three independent experiments. MGE, medial ganglionic eminence; Fc, Fragment crystallizable ; PLL, poly-L-lysine.

3.2.2 Sema3C-AP gradient chemoattracts neurons derived from medial ganglionic eminences (MGEs).

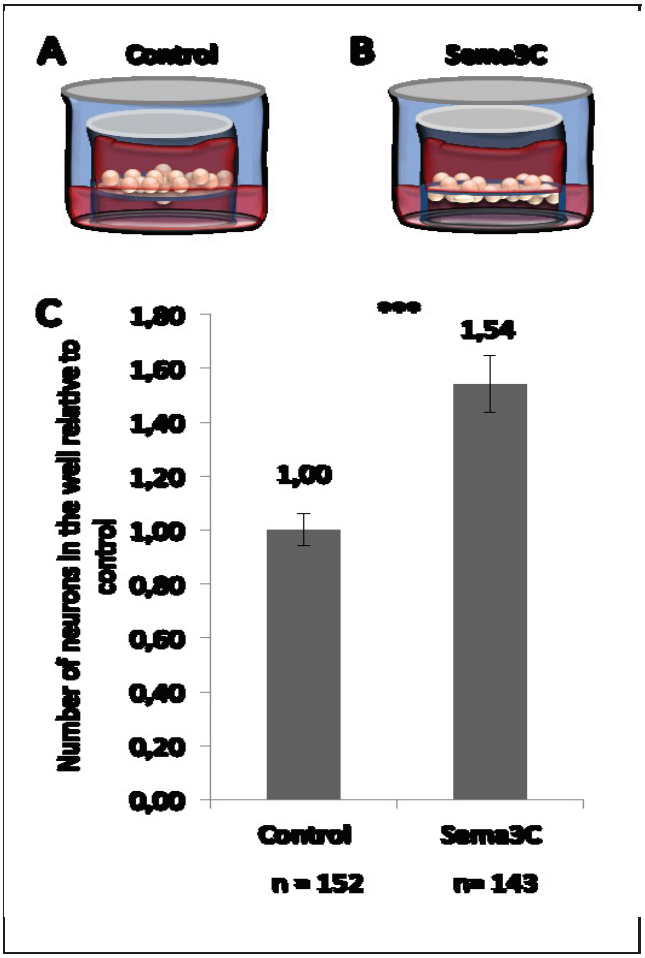
The data obtained from the stripe assay described in the section 3.2.1 suggested that Sema3C-Fc might be an attractive cue for MGE-derived neurons. The stripe assay simulated membrane bound guidance cues, since the stripes contained immobilized Sema3C-Fc bound to the coverslip. But Sema3C is a secreted protein and diffuses through the pathway of migrating interneurons (Bagnard, Thomasset et al. 2000). To clarify if Sema3C-AP gradients guide cortical interneurons, Boyden chamber assays were implemented.

The Boyden chamber assay uses inserts containing a permeable membrane with 8 µm pores pre-coated with collagen. The membrane serves as a barrier and creates an upper and lower compartment that can be filled with different media. Generally, the migratory cells are place in the upper compartment and the medium containing the cue of experimental interest is placed in the lower compartment (Fig. 18 A, B). The protein of interest diffuses and creates a gradient through the membrane. Migratory cells are able to move through the membrane pores towards the medium of preference. As a result, when exposed to chemoattractant gradients, the cells migrated to the bottom side of the membrane, towards the increasing

gradient (Fig. 18 B). On contrary, when exposed to chemorepellent gradients, the cells rather settled on the top, were the concentration of the tested guidance cue decreases.

For performing the Boyden chamber assay, the lower compartment of each insert was filled either with control or Sema3C-AP conditioned media (Section 2.2.4.4) that diffused and created a gradient. Next, dissociated E14.5 MGE neurons were prepared (Section 2.2.2.3), and plated in the upper compartment. After 6 h in vitro, the membrane with the cells were fixed and stained with DAPI. Subsequently the number of cells that migrated through the membrane towards the lower compartment was determined. The amount of cells migrating under Sema3C-AP conditions were standardized in relation to the control.

As illustrated in Figure 18 C, the relative number of migrating cells increased about 50 % when the lower compartment contained Sema3C-AP compared with control conditions (Control 1 ± 0.06 ; Sema3C-AP 1.5 ± 0.11 ; Control n = 60 frames analyzed; Sema3C-AP n=60 frames analyzed; Student´s t-test; *** $p \leq 0.001$). Consequently, MGE dissociated cells migrate towards an increasing gradient of Sema3C-AP, indicating that Sema3C acts as an attractive guidance cue.



ure 18. Sema3C-AP gradient has an tractive effect on MGE neurons in the yden chamber assay. (A, B) Scheme of the serts used in the Boyden chambers assay. 4.5 MGE single cells were placed in the per compartment of the chamber, the cue sted in the lower section. Single cells were cubated for 6 h. (B) Migratory cells are able extend protrusions towards the gradient of tracellular cue in the medium, via actin toskeleton reorganization, and ultimately ss through the pores of the polycarbonate embrane in the presence of an attractive e. (A) In the case of a neutral cue placed in e lower compartment, cells will pass less rough the membrane. (C) Bars show the antification of neurons in the lower well ative to control. Values showed that ma3C-AP gradients exerted attraction on GE-derived cells. n , number of frames alyzed; Student´s t-test: ***p≤ 0.001, error rs are SEM. Results are from two dependent experiments.

3.2.3 Sema3C-AP gradient attract neurons derived from the ventricular/sub ventricular zone (VZ/SVZ) of medial ganglionic eminences (MGEs).

It has been shown that depending on the domain of origin, cortical interneurons follow two different pathways to the neocortex (Marin et al. 2001, Zimmer et al. 2011). Cortical interneurons derived from the POA mostly follow the “superficial migratory stream” and expressed, among others, Neuropilin 2 (Nrp2) receptors. On the other hand, cortical interneurons derived from the MGE mainly reach the cortex through the “deep migratory stream” along the VZ and express Neuropilin 1 (Nrp1) receptors. Both Nrp1 and Nrp2 have been described as possible interaction partners for Semaphorin 3C (Castellani et al. 2004, Ruediger, Zimmer et al., 2013).

In sections 3.2.1 and 3.2.2, the E14.5 medial ganglionic eminences (MGE) were dissected and MGE-derived neurons were equally distributed in the stripe and Boyden chamber assay. Results on both sections showed that substrate bound Sema3C-Fc on the stripe assay and secreted Sema3C-AP gradients in Boyden chamber assays, have an attractive effect on single neurons. In order to test the effect of secreted Sema3C-AP gradient in experiments that closely resembles in vivo conditions and to study if Sema3C attracts neurons following a specific migratory stream, E14.5 explants were prepared separately from specific parts of the MGE. Dissecting the VZ of the MGE, explants that contain the deep migratory stream were obtained. Dissecting the IMZ of the MGE, explants that contain part of the superficial migratory stream were obtained (Fig. 18). The explants were cocultured 2 DIV in a three-dimensional matrix of chicken plasma coagulated with thrombin, with HEK cells aggregates that secrets continuously Sema3C-AP, Sema3A-AP or control proteins that diffused through the chicken plasma matrix and created a gradient. Therefore the area close to the HEK cell aggregate was more exposed to the recombinant proteins than the areas away.

Sema3A-AP was used to further analyze its repellent effect on MGE-derived migrating interneurons. In vivo, Sema3A is secreted by cells in the striatum and have been shown to be repulsive for migrating cortical interneurons preventing from innervating this developing structure (Marin et al., 2001; Hernández-Miranda et al., 2011; Zimmer et al., 2010).

After 2 DIV coculture and further fixation, the analysis of the explants with neurons deriving from all around the surface of the explants (Fig. 19), were performed. Firstly, the growth of the explants was scored. For this, explants were considered as 0 when neurons migrated uniformly all over the explant, as 1 or 2 when neurons grew moderate or strongly towards

the HEK cell aggregate and as -1 or -2 when neurons migrated moderate or strongly away from the HEK cell aggregate (Section 2.4.4.5.1, Fig. 10). After scoring, a guidance index was calculated averaging all the score values of the explants for every condition.

As expected, neurons derived from VZ/SVZ explants or IMZ explants of the MGE confronted with aggregates of HEK untransfected cells (control conditions) migrated uniformly in all directions. A guidance index of -0.05 ± 0.10 for VZ/SVZ explants and 0.00 ± 0.30 for IMZ explants were obtained when cocultured MGE-explant with untransfected HEK cells (Fig. 19 B, C). In the presence of Sema3A-AP secreting cell aggregates, neurons from VZ/SVZ explants and IMZ explants of the MGE migrated away from the guidance cue, obtaining a negative index of -0.67 ± 0.21, and -1 ± 0.04 respectively. These results indicated that Sema3A-AP gradients are repellent for migrating cortical interneurons presents in the VZ/SVZ and IMZ (Fig. 19 B, C; VZ/SVZ: Control n = 55, Sema3A-AP n = 18; Student´s t-test, ** $p \leq 0.01$; IMZ: Control n = 8, Sema3A-AP n = 6; Student´s t-test, * $p \leq 0.05$). Such repellent effect has been already described for E13.5 MGE neurons exposed to Sema3A in previous works (Marin et al., 2001; Hernández-Miranda et al., 2011; Zimmer et al., 2010).

In contrast, in the presence of Sema3C-AP secreting cell aggregates neurons from VZ/SVZ explants of the MGE migrated more towards the source of the Sema3C gradient, (Fig. 19 B) obtaining a guidance index of 0.90 ± 0.14 (Fig. 15 B; VZ/SVZ: Control n = 55, Sema3C-AP n = 20; Student´s t-test , *** $p \leq 0.001$). These results confirmed that Sema3C might play a role in guiding cortical interneurons.

Sema3C-AP seems to have no effect on neurons from the IMZ. Explants from the IMZ that were cocultured with Sema3C-AP expressing HEK cells revealed a migration index of 0.00 ± 0.25 (Fig. 19 C; IMZ: Control n = 8, Sema3C-AP n = 16; Student´s t-test, n.s). The results suggest that Sema3C have no effect on interneurons en route to the cortex through the superficial migratory stream.

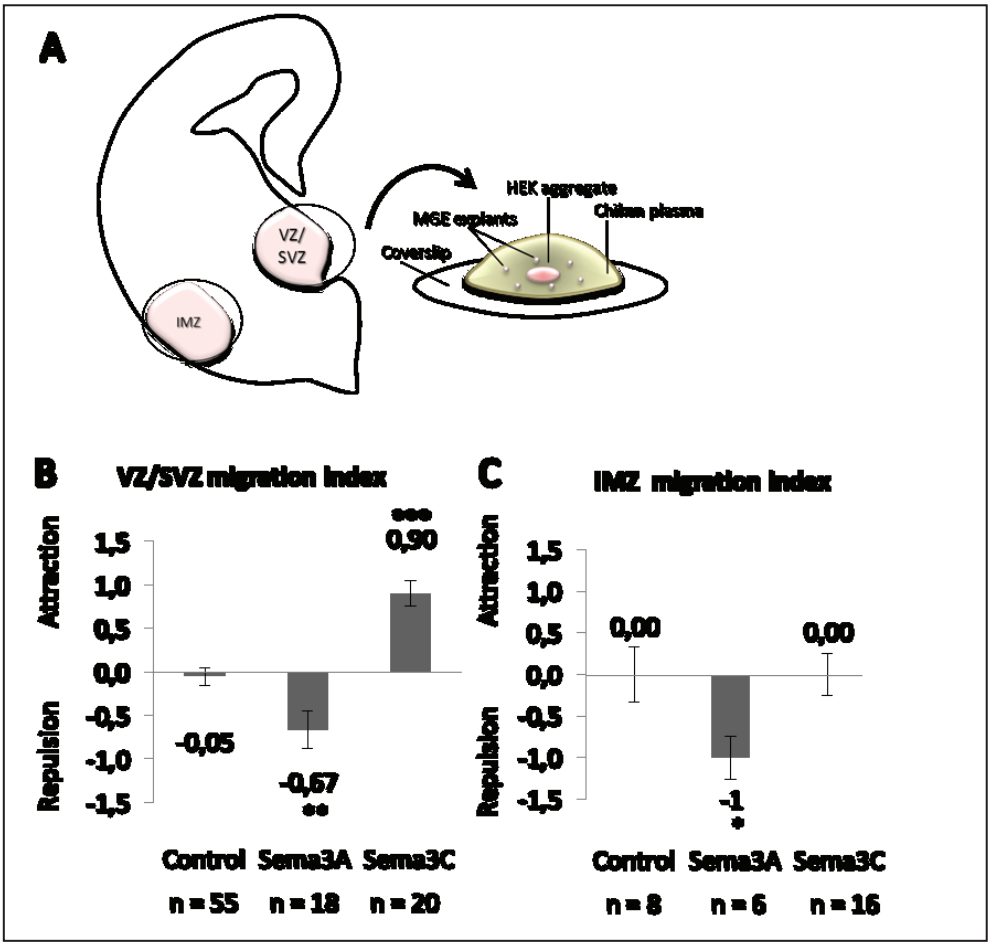


Figure 19. Response of explants from the VZ/SVZ or IMZ of the MGE to Sema3A-AP, Sema3C-Ap or control gradients in coculture assays. (A) Experimental design, E14.5 coronal slices were used to dissect VZ/SVZ or IMZ of MGEs and form explants. One HEK aggregate and 5 to 7 explants were cocultured 2 DIV in chicken plasma cross-linked with thrombin. (B-C) Quantification of the guidance index for VZ/SVZ or IMZ explants. Bar shows the guidance index values under each coculture condition. Neurons derived from the VZ/SVZ of the MGE are repelled by Sema3A and attracted towards Sema3C. In contrast, neurons derived from the IMZ of the MGE were repelled by Sema3A, whereas Sema3C had no effect on them. n, explants analyzed; Students t-test: ***p < 0.001; error bars are SEM. Results are from at least three independent experiments using VZ/SVZ explants and two independent experiments using IMZ explants. VZ/SVZ, Ventricular/subventricular zone; IMZ, Intermediate zone; MGE, medial ganglionic eminences ; HEK, Human embryonic kidney cells.

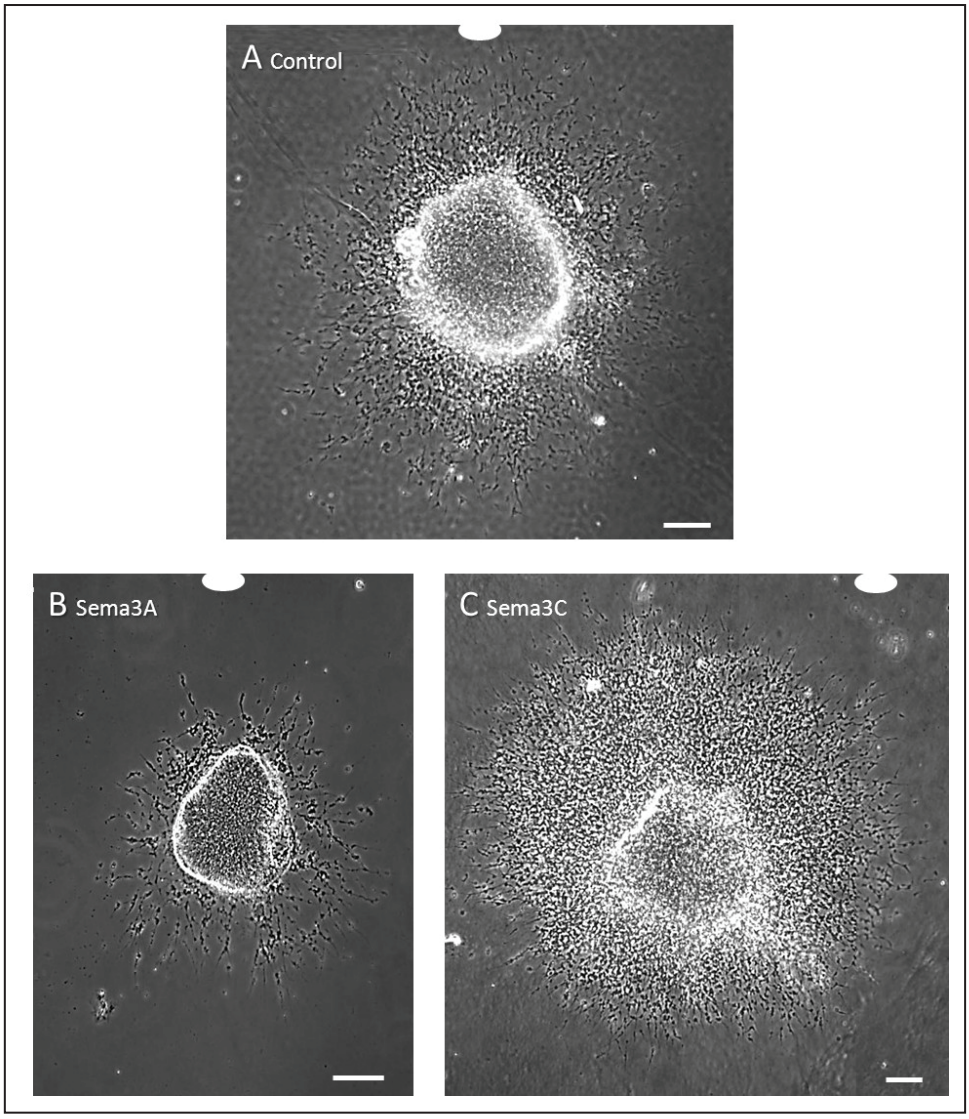


Figure 20. Response of explants from the VZ/SVZ of the MGE to Sema3A-AP, Sema3C-Ap or control gradients in coculture assays. E14.5 coronal slices were used to dissect VZ/SVZ MGEs and form explants. One HEK aggregate and 5 to 7 explants were cocultured 2 DIV in chicken plasma cross-linked with thrombin. (A-C) Micrographs of representative MGE explants cocultured with (A) aggregates of non-transfected HEK cells (control), (B) Sema3A-AP transfected HEK cells and (C) Sema3C-AP transfected cells. White oval represent the position of the HEK aggregates. Scale bars: 100 µm.

Secondly, another approach was used to quantify and analyze if gradients of Sema3C affect the migration of cortical interneurons. Because Sema3C-AP appears to have no effect on interneurons from the IMZ, only the explants from the VZ/SVZ of the MGE were used. Initially, in every explant analyzed, the proximal and distal area in relationship to the HEK cell aggregate were marked (Fig. 20). Then, in each area the distance between the clear border of the explants and the soma of the ten farthest neurons out of the explants were measured, as illustrated in Figure 20.

As expected, explants from the VZ/SVZ of the MGE confronted with untransfected HEK cell aggregates migrated out equal distances on each area, with an average value of 229.41 ± 6.73 µm. In contrast, explants opposed to Sema3A-AP gradients migrated 30 % less in the proximal area than under control conditions, with an average distance of 166.93 ± 12.60 µm. In the distal area, were the amount of Sema3A-AP protein is more diluted, neurons migrated 50 % further compared to control conditions, indicated by an average distance of 344.46 ± 37.21 µm (Fig. 21; Control n = 420, Sema3A-AP n = 60 proximal area, Sema3A-AP n = 60 distal area, Student´s t-test, *** $p \leq 0.001$).

In contrast, on explants from the VZ/SVZ of MGEs, confronted to Sema3C-AP gradients, the neurons in the proximal area migrated 36 % further than under control condition, with an average distance of 310.52 ± 14.23 µm (Fig., 21; Control n = 420, Sema3C n = 130 proximal area; Student´s t-test, *** $p \leq 0.001$). In the distal area, were the amount of Sema3C-AP protein is more diluted, the migrated distances were similar to control conditions, with an average value of 224.23 ± 14.50 µm (Fig., 21; Control n = 420, Sema3C-AP n = 130 distal area; Student´s t-test, n.s.).

In summary, the results confirm a repellent effect of Sema3A-AP on cortical interneurons derived from the VZ/SVZ of the MGE. The effect of Sema3A-AP seems to be stronger when the neurons are exposed to a decreasing Sema3A-AP gradient, indicating that the protein concentration modulates the effect of Sema3A on cortical interneurons. Furthermore, the effect of Sema3C-AP seems to be concentration dependent as well. Sema3C-AP appears to have an attractive effect on cortical interneurons since the interneurons migrated father distances when exposed to a higher Sema3C-AP protein concentration.

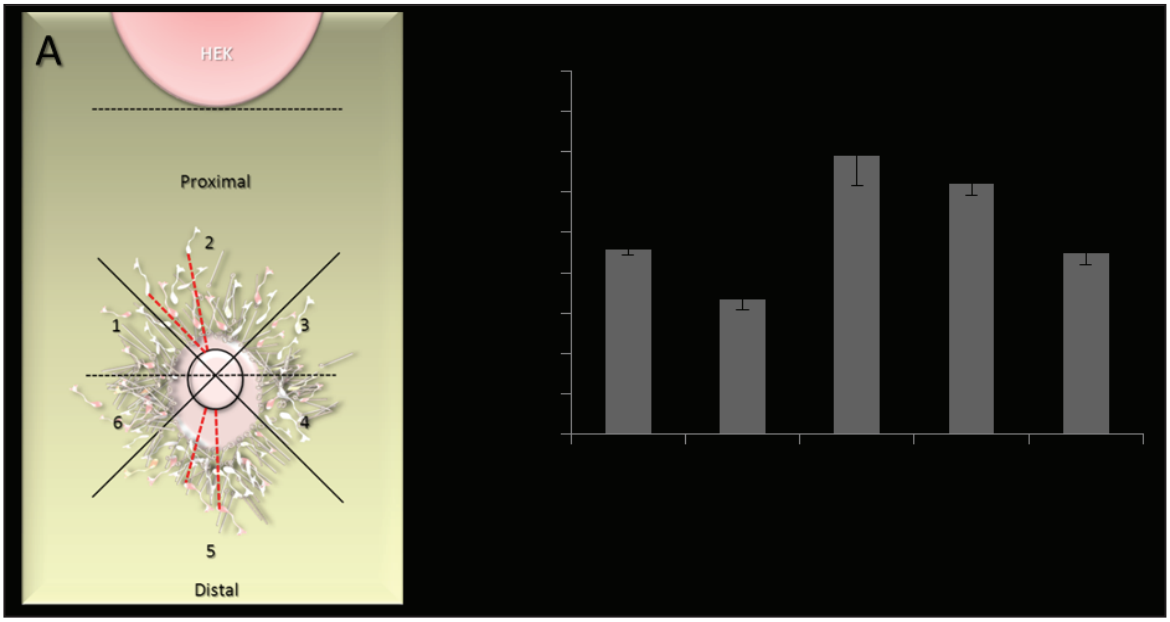


Figure 21. Interneurons derived from the VZ/SVZ of MGE, migrated different distances depending on the presence of Sema3A-AP, Sema3C-AP or control gradients. (A) Design of the experimental quantification. E14.5 coronal slices were used to dissect VZ/SVZ of MGEs and form explants. One HEK aggregate and 5 to 7 explants were cocultured 2 DIV in chicken plasma cross-linked with thrombin. Analysis was performed after 2 DIV coculture, and further fixation, for every condition blindly. The initial shape of the MGE was outline with a black line. Proximal and distal areas were set perpendicular to the HEK cell aggregate. In the distal area, dashed red lines illustrate the migration distance of two MGE-derived interneurons, measured from the initial border of the MGE explant to the soma. (B) Bar shows the distance migrated by the MGE neurons on each coculture condition. Neurons migrated shorter distances towards Sema3A increasing gradients, whereas migrated longer distance away from Sema3A decreasing gradients. In contrast, neurons migrated longer distances towards Sema3C increasing gradients, while decreasing Sema3C gradients had no effect on their migration when compared to control conditions. Students t-test: ***$p < 0.001$; error bars are SEM. Explant used for analysis had a clear initial border. Results are from at least three independent experiments.

3.2.4 Sema3A-AP and Sema3C-AP effects in the neurites and growth cone of neurons derived from the medial ganglionic eminences.

Recently, some studies have tried to clarify if guidance cues influence the morphology of cortical interneurons neurites. For example, migrating interneurons exposed to repulsive cues seem to suppress neurite growth, (Zhu et al., 1999; Sang et al., 2002).

Changes in the morphology of axons, especially in their processes and growth cones have been often described. On contrary, much more needs to be studied concerning changes in the morphology of migrating interneurons. For example, one characteristic broadly studied on axons but not on migrating interneurons, is the shape of the growth cone. Under control conditions, axonal growth cones possess spread morphology. This characteristic relates to the growth cone screening of guidance cues from its environment. On contrary, if axonal

growth cones come across a repellent cue it will retract, presenting a collapsed morphology, generally more long than wide (Ox et al., 1990). Such observations on axonal growth cones might apply for growth cones of migrating interneurons, since both structures seem similar. For example both often recognize and react to signaling molecules on their surroundings (Castellani and Rougon 2002, Raper and Mason 2010).

Common ways to study the morphology changes mentioned above, is performing coculture or collapse assays. The collapse assay consists on culturing single axons and exposed them to media with the signal molecule to be tested. After generally 2 h exposure, the length of the axons and growth cones are examined using time lapse or micrographs. Next, with programs like ImageJ the length of the axons, the area, length and width of the growth cone are measured. So that any change on the axonal morphology can be register.

The effects of Sema3A and Sema3C have been described for cortical axons in coculture experiments. Sema3A-AP gradients reduced the axonal length of fibers growing towards the aggregate with increasing Sema3A-AP concentration (Bagnard et al., 1998, Ruediger, Zimmer et al., 2013). In contrast, Sema3C-AP gradients increased the axonal length of fibers growing towards its source (Bagnard et al., 1998; Ruediger, Zimmer et al., 2013). Also, it has been described growth cone collapse for cortical axons under Sema3A-AP gradients, since its growth cone were reduced when compared to control conditions. In contrast, when exposed to Sema3C-AP no effect was observed on the axonal growth cones (Bagnard et al., 1998). Since the effect of Sema3C and Sema3A on interneuron morphology remains to be elucidated, in the present study, coculture and collapse assays were performed using neurons derived from the VZ/SVZ of E14.5 MGEs.

First of all, coculture experiments were done as described in Section 2.4 (Fig. 22 A). For analysis, the processes lengths of migrating neurons were measured only in the proximal and distal areas in relation to the HEK aggregate position (Sectors 2 and 5; Fig. 22 B, Described in Section 2.4.1.5). Thus, the processes from the ten farthest neurons that migrated out of the explant were measured from the base of the soma to the growth cone of the neurite. The analyzed neurites had no contact with other cells.

After analysis, under control conditions, no significance differences were observed between neurite lengths of proximal compared to distal areas (Fig. 22 C). Therefore the mean length of the processes was calculated with the measures from both areas together: 45.72 ± 1.10°µm. In contrast, under the influence of Sema3A-AP gradient, the results showed

shorter neurites in the proximal area with an average value of 32.12 ± 2.67 µm compared to the distal area 45.59 ± 2.01 µm and to control conditions 45.72 ± 1.10 µm (Fig. 22 C; Control n = 400, Sema3A-AP n = 60 proximal area, Sema3A-AP n = 60 distal area; Student´s t-test, *** $p \leq 0.001$). Next, whiting Sema3C-AP gradients, the mean process length remains similar to control in the proximal and distal areas with a value of 46.02 ± 1.18 µm and 44.70 ± 1.89 µm respectively (Control n = 400 , Sema3C-AP n = 130 proximal area, Sema3C-AP n= 130 distal area; Student´s t-test, n.s.).

In summary, this analysis suggested that Sema3A-AP gradients affected neurite morphology by inhibiting its outgrowth, only when the concentration of the protein is increasing. However, Sema3C-AP gradients do not seem to affect neurites morphology on neurons from the VZ/SVZ of E14.5 MGEs.

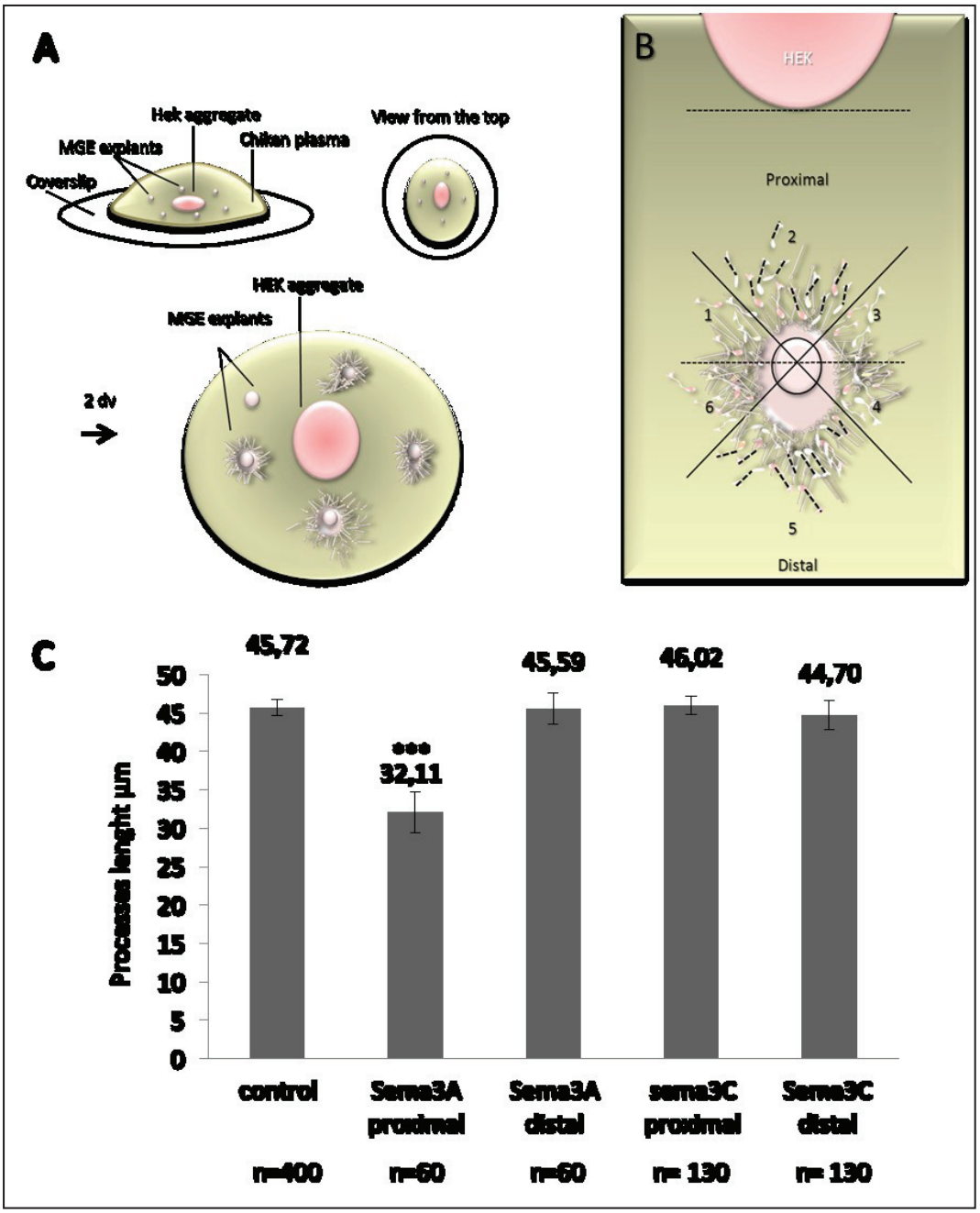


Figure 22. The processes length of migrating neurons is affected only by increasing Sema3A-AP and not by Sema3C-AP gradients. (A) Experimental design. E14.5 coronal slices were used to dissect VZ/SVZ of MGEs and form explants. One HEK aggregate and 5 to 7 explants were cocultured 2 DIV in chicken plasma cross-linked with thrombin. Analysis was performed after 2 DIV coculture, and further fixation, for every condition blindly. (B) The initial shape of the MGE was outline with a black line. Proximal and distal areas were set perpendicular to the HEK cell aggregate. Dashed lines represent the length of the neuronal processes. (C) Quantification of the process length. Bars show the length of the migrating neurons on each area for each coculture condition. Only when neurons confronted increasing Sema3A gradients the length of their neurites was reduced, when compared with control conditions. Student´s t-test: ***p< 0.001; error bars are SEM. Explant used for analysis had a clear initial border. Results are from at least three independent preparations.

Next, collapse assays were performed, where E14.5 MGE-derived neurons were plated, cultured 46 h and exposed for 2 h to tenfold control, Sema3A-AP or Sema3C-AP conditioned media. After fixation, the neurons were stained with phalloidin for marking the F-actin and visualize the morphology of their tips. The growth cone response was quantified by measuring its area (Fig. 23) as well as the width and length (Fig. 23 E).

After analysis, on control conditions (Fig. 23 A) an average area of 25 ± 2.00 µm2 was obtained. After 2 h exposure to Sema3A-AP, neurons from the VZ/SVZ of the E14.5 MGE increased its growth cone area, indicated by an average value of 47 ± 3.23 µm2 (Fig. 23 B, D; Control n = 47 , Sema3A-AP n = 62; Student´s t-test, *** $p \leq 0.001$). In contrast, no effect was observed under Sema3C-AP conditions (Fig. 23 C, D) with an average area of 31 ± 2.59 µm2 (Control n = 47, Sema3C-AP n = 58, Student´s t-test, n.s.).

Concerning the length and width of the growth cone, under Sema3A-AP conditioned media, the average values showed an increase: 10.94 ± 0.41 and 7.26 ± 0.44 µm respectively compared to 7.82 ± 0.38 µm and 5.21 ± 0.37 µm under control conditions (Fig. 23 F; Control n = 47, Sema3A-AP n = 62, Student´s t-test ** $p \leq 0.01$; *** $p \leq 0.001$). This increase was in a proportional way, and suggested a spread shape of the growth cone. Under Sema3C-AP conditions there was a significant change in the length of the growth cone but not in the width, represented by values of: 9.4 ± 0.45 µm and 5.34 ± 0.39 µm respectively (Control n°=°47, Sema3C-AP n = 59, Student´s t-test * $p \leq 0.05$, n.s.).

In brief, Sema3A-AP gradients affected the morphology of migrating neurons, indicated by a significant reduction on the average length of the processes. Such reduction occurred only when the concentration of the protein was increasing in the area towards the source of Sema3A-AP. Furthermore, a significant increase of the average area of the growth cone was registered suggesting spread morphology. On contrary, when exposed to Sema3C-AP only an enlargement of the growth cone length was observed. No changes on the length of the processes were registered under the influence of Sema3C-AP gradients.

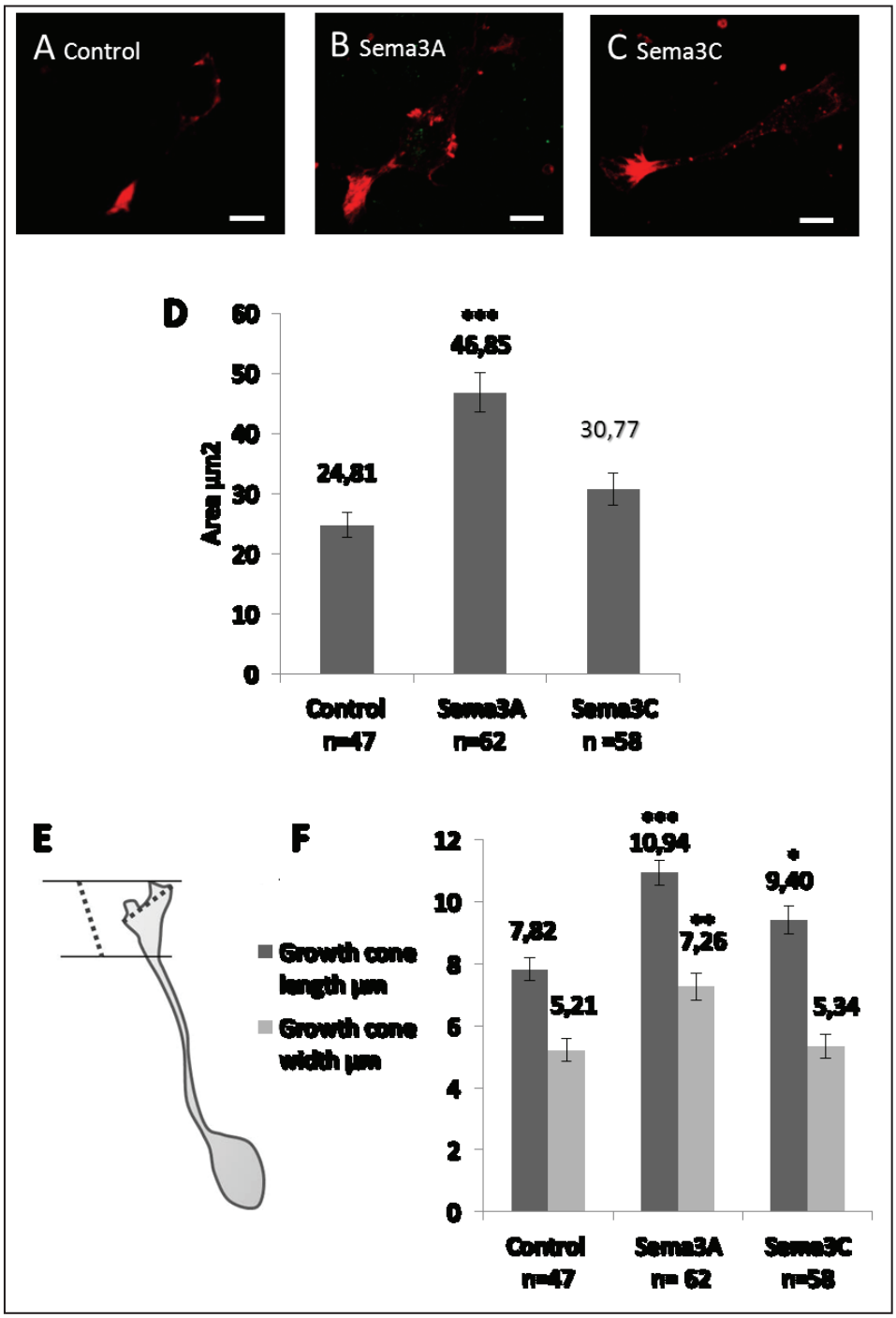


Figure 23. Effect of Sema3A-AP or Sema3C-AP recombinant proteins on growth cone of MGE neurons. E14.5 MGE single cells were culture for 46 h and expose during 2 h to tenfold control, Sema3A-AP or Sema3C-AP conditioned media. Neurons were fixed and stained with phalloidin in order to visualize the cytoskeleton. (A-F) Quantification of the area of neuronal growth cone. To quantify the effect of the different proteins tested, the area of the growth cone and the length and width was measured using the ImageJ program. (A-C) Micrograph of neurons with: (A) control conditioned media, (B) Sema3A-AP conditioned media (C) Sema3C-AP conditioned media (D) Bars show the growth cone area in µm2 of the leading process of single neurons for each culture condition (E) Diagram of the measurement. (F) Quantification of the width and length of the growth cones. Bars show the growth cone length and width of single neurons for each culture condition. n, number of neurons analyzed; Student T-test with Bonferroni correction. Error bars are SEM Results are from two independent experiments. Scale bars equals 10 µm.

Chapter 4: Discussion

Cortical interneurons derived from the basal telencephalon and migrate long distances to their final position in the developing cortex (Anderson, Marin et al. 2001, Hansen, Lui et al. 2013, Ma, Wang et al. 2013, Molnar and Butt 2013). Compiling evidence points out that transcription factors not only define interneuron differentiation but also prespecify them by modulating their signaling receptors (Nobrega-Pereira and Marin 2009, McKinsey, Lindtner et al. 2013, van den Berghe, Stappers et al. 2013, Guo and Anton 2014). Such signaling receptors allow migrating interneurons to respond selectively to environmental cues. Thus, cell intrinsic programs together with guiding molecules assure that cortical interneurons reach their target areas (Flames, Pla et al. 2007, Nobrega-Pereira and Marin 2009). Several classes of guidance molecules, including Semaphorins, have been implicated in the regulation of interneuron migration. Guidance cues create permissive and non-permissive environments used by cortical interneurons to enter the neocortex (Marin, Yaron et al. 2001, Marin and Rubenstein 2003, Wichterle, Alvarez-Dolado et al. 2003, Flames, Long et al. 2004, Guo and Anton 2014). Among the Semaphorin family, it has been shown that Sema3A and Sema3F are expressed in the striatum and repel migrating cortical interneurons from entering into this developing structure. On this way, the striatum is innervated only by striatal interneurons, whereas cortical interneurons move forward to their target areas in the dorsal telencephalon (Marin, Yaron et al. 2001, Nobrega-Pereira, Kessaris et al. 2008).

In the present study, the repulsive effect of Sema3A on interneuron migration was studied in more detail. A major focus, however, was to examine the potential role of Sema3C on interneuron migration. This was motivated by previous results showing that Sema3C expression patterns sharply demarcate the pallial/subpallial border and the intracortical pathway of migrating interneurons in the subventricular zone of the neocortex (Bagnard, Lohrum et al. 1998, Ruediger, Zimmer et al. 2013). Another fact that prompted the study of Sema3C is that, for cortical axons, Sema3C acts as an attractive cue (Bagnard, Lohrum et al. 1998, Ruediger, Zimmer et al. 2013). Furthermore, Sema3C is a secreted protein and may form a gradient that reach cortical interneurons earlier on their route from the basal telencephalon towards the cortex. Moreover, migrating cortical interneurons express Nrp1 and Nrp2, possible Sema3C receptors. All these facts lead to the hypothesis that Sema3C might also attract migrating interneurons. As it will be discuss in more detail below,

experiments from different in vitro assays indicated that Sema3C might indeed be an attractive cue for a subpopulation of cortical interneurons.

4.1 Sema3A is a repulsive guidance molecule for MGE-derived interneurons during tangential migration.

Among the Semaphorin guidance molecules, for migrating MGE-derived cortical interneurons, only a repellent effect by Sema3A and Sema3F have been shown (Marin, Yaron et al. 2001, Zimmer, Schanuel et al. 2010, Hernández-Miranda 2011). Sema3A and Sema3F are secreted proteins and are likely to form gradients in vivo, it remains unclear how the gradients of such diffusible proteins affect migrating cortical interneurons. Using coculture assays, the present study confirms the repellent effect of Sema3A and additionally clarifies how Sema3A gradients affect migrating MGE-derived interneurons.

The coculture assay allows the study of cells exposed to increasing and decreasing gradients, in this case of the diffusible guidance cue Sema3A. Previous studies have shown that increasing concentrations of Sema3A repelled cortical axons (Bagnard, Lohrum et al. 1998, Bagnard, Thomasset et al. 2000). Specifically, increasing gradients of Sema3A caused growth cone collapse, accompanied by a reduced axonal length. In contrast, no effect was seen on growing cortical axons exposed to decreasing concentrations of Sema3A (Bagnard, Lohrum et al. 1998, Bagnard, Thomasset et al. 2000).

As expected, interneurons from the ventricular and subventricular zone of the medial ganglionic eminences (VZ/SVZ of the MGEs) migrated shorter distances when exposed to increasing concentrations of Sema3A, in comparison to interneurons under control conditions. It seems likely that interneurons encountering an increasing Sema3A gradient stop moving towards such a repellent environment. Interestingly, interneurons from the VZ/SVZ of the MGE also were repelled by decreasing Sema3A gradients and migrated far towards the area with less concentration of Sema3A. As mentioned before, Sema3A is secreted in the striatum, diffuses and generates a gradient in vivo. Therefore, the present results implicate that, in vivo, not only the striatum would be free of cortical interneurons, but these interneurons might be stimulated by the decreasing concentration of Sema3A to keep on moving towards the neocortex.

Two models have been proposed for interneuron migration and reorientation when encountering guidance cues. One model suggests that migrating neurons with a bipolar

morphology, sense with their growth cone guidance cues. When expose to repulsive cues like Slit-2, neurons increase their growth cones Ca^{2+} levels (Guan, Xu et al. 2007). This change in the intracellular Ca^{2+} concentration leads to a rearrange in the distribution of the Rho A protein (Ras homolog gene family, member A). Thus, in the neuron, Rho A circulates from the growth cone to the soma causing a growth cone collapse, similar to what have been described for extending axons (Liu and Strittmatter 2001, Guan, Xu et al. 2007, Bashaw and Klein 2010). Next, the area where the Rho A concentration increased is the new leading front, where the leading process will be develop (Guan, Xu et al. 2007). Migration and reorientation base on changes of the intracellular calcium levels have been describe on granular neurons from the cerebellum and some evidence points that similar mechanism affects migrating cortical interneurons (Metin, Denizot et al. 2000, Bortone and Polleux 2009).

Another study described that interneurons responding to guidance cues generated branches, a mechanism that allows them to explore a broad territory (Martini, Valiente et al. 2009, Lysko, Putt et al. 2011). The growth cone of each branch is likely to recognize various concentrations of molecule or even a different set of guidance signals. Thus, the interneuron stabilizes the branch positioned towards the most suitable environment. When an interneuron branch turns into the leading process, it rapidly changes the morphology of its growth cone. Specifically, the stabilized leading process displays an elaborate growth cone, whereas the transient branch retracts (Bellion, Baudoin et al. 2005, Kappeler, Saillour et al. 2006, Martini, Valiente et al. 2009). It is likely that interneurons subpopulation use one form to reorient or migrate or even that interneurons switch among both mechanisms.

According to Marin et al (2001), Sema3A is repellent for cortical interneurons derived from the ventricular and subventricular zone of the medial ganglionic eminences of the basal telencephalon. It is worth noting that around the developmental day E14.5, the VZ/SVZ of the MGE generates cortical interneurons that mainly follow the deep migratory stream. In contrast, the preoptic area (POA) generates cortical interneurons that mostly follow the superficial migratory stream and pass by the intermediate zone (IMZ) of the MGE (Marin and Rubenstein 2003, Zimmer, Rudolph et al. 2011, Arber and Li 2013). Therefore, to test the response of other subpopulations of cortical interneurons to Sema3A gradients, coculture assays using IMZ explants were performed. Interestingly, Sema3A also repelled interneuron

subpopulations contained in the IMZ. In contrast, as will be discuss later, Sema3C had no effect on interneurons located in the IMZ.

It has been proposed that Sema3A repulsion on migrating cortical interneurons was likely mediated by Nrp1 receptors (Marin, Yaron et al. 2001, Zimmer, Schanuel et al. 2010). The present study showed that Sema3A also repels cortical interneurons migrating through the IMZ, in the superficial migratory stream. It have been described that interneurons contained in the IMZ mainly express Nrp2 receptors and not Nrp1 (Marin, Yaron et al. 2001, Nobrega-Pereira, Kessaris et al. 2008, Zimmer, Rudolph et al. 2011). Thus, it would be interesting to study which receptors are used by this subpopulation of interneurons for Sema3A signal. Moreover, other molecules could interact with the receptor. For example, it have been described that Robo1-Nrp1 or LIM-kinase 2 (Limk2)-PlexinA1 interaction, indirectly modulate Sema3A repulsion on migrating MGE-derived cortical interneurons (Hernández-Miranda 2011, Andrews, Zito et al. 2013).

A novel result from the present study relates to changes in the morphology of interneurons after exposure to Sema3A. Several years ago the growth cone collapse assay lead to the discovery of Sema3A, previously named “colapsin” (Luo, Raible et al. 1993). Such effects illustrate how guidance cues coordinate cytoskeletal rearrangements necessary for reorienting axonal navigation. So far, it has been described that Sema3A signal regulates Rho GTPases, including Rho and Rac molecules and associated proteins (Jin and Strittmatter 1997, Vastrik, Eickholt et al. 1999, Nakamura, Kalb et al. 2000, Liu and Strittmatter 2001, Castellani and Rougon 2002). In general, the activation of Rho proteins enhances the contractility of actomyosin, causing retraction of the growth cones, whereas active Rac proteins boost the spreading of growth cones (Kruger, Aurandt et al. 2005). Recently, another mechanism for the growth cone collapse induced by Sema3A has been proposed. Zylbersztejn et al., (2011) found that the vesicular SNARE protein Synaptobrevin 2 causes massive endocytosis of the axonal membrane bearing Nrp1-PlexA1 heterodimers bound to Sema3A on cortical axons (SNARE proteins are “exocytic vesicular soluble N-ethylmaleimide sensitive fusion protein attachment protein receptor”). This endocytosis results in the shrinking of the growth cone, preventing the progression of the growth cone towards the repellent Sema3A source. Thus, the turn of the axon away of Sema3A is favor. Such endocytosis related to Sema3A growth cone collapse have been described in cortical, retinal and dorsal root ganglion axons (Fournier, Nakamura et al. 2000, Castellani, Falk et al. 2004,

Piper, Salih et al. 2005, Zylbersztejn, Petkovic et al. 2012). Further studies can clarify if each mechanism described for Sema3A induced growth cone collapse is specific for each type of axons, or that both mechanism work in conjunction.

In the present study, the collapse assay was used to examine if Sema3A also induces growth cone collapse on cortical interneurons. Migrating neurons and extending axons possess growth cones and often respond to the same guidance cues (Tessier-Lavigne and Goodman 1996, Komuro, Yacubova et al. 2001, Nasrallah, McManus et al. 2006, Martini, Valiente et al. 2009, Gopal, Simonet et al. 2010). Since migrating neurons and extending axons are both repelled by Sema3A gradients, it is possible that their sensing and responding to this guidance cue is mediated by similar mechanisms. Interestingly, instead of a growth cone collapse, Sema3A exposure results in an enlargement of the growth cone area on interneurons, compared with control conditions. Obviously, although extending axons and migrating neurons are repelled by Sema3A, their growth cones respond in different ways.

The enlargement in the growth cone area of interneurons exposed to Sema3A could be explained by the way interneurons reorient when encountering guidance cues. Sema3A repelled migrating interneurons causing that their more suitable branch turned into a leading process and therefore exhibit an increased growth cone area more often than under control conditions. Another possibility relates with the signaling cascade activated by Sema3A binding. On extending axons and migrating cortical interneurons, Sema3A activates Rho GTPases, but on axons Rho proteins might be activated, generating growth cone collapse, whereas on interneurons other downstream Rho GTPases protein, like Rac, might be activated, causing the spreading on their growth cone (Kruger, Aurandt et al. 2005).

In addition, coculture assays could show that interneurons derived from the VZ/SVZ of the MGE have shorter processes when exposed them to increasing gradients of Sema3A. As mentioned before, previous studies found a reduction in the length of cortical axons exposed to increasing concentrations of Sema3A. However, decreasing concentrations of Sema3A have no effect on growing cortical axons (Bagnard, Lohrum et al. 1998, Bagnard, Thomasset et al. 2000, Zylbersztejn, Petkovic et al. 2012). Regarding interneuron processes, no information is available about length modifications after Sema3A exposure. Nevertheless, shorter neurites is a feature already described for interneurons encountering repellent cues like Slit1 (Hu 1999, Sang, Wu et al. 2002). In contrast, longer neurites during interneuron

migration have been related with a decreased motility (Yamasaki, Tanaka et al. 2010, Inamura, Kimura et al. 2012, Steinecke, Gampe et al. 2012).

The reduction in the length of interneuron processes after Sema3A exposure could be due to modifications in the extent of time interneurons take to move. Three steps have been described for interneuron movement (Ayala, Shu et al. 2007, Martini, Valiente et al. 2009). First, interneurons extend a leading process and usually generate branches that allow interneurons to reorientate. Both leading process and branches are extended towards the front of the neuron and have a dynamic, exploratory behavior (Bellion, Baudoin et al. 2005, Kappeler, Saillour et al. 2006, Martini, Valiente et al. 2009). Second, the interneuron decides which direction to follow. At this point, only one of the branches is stable and becomes the leading process, whereas the other retracts (Bellion, Baudoin et al. 2005, Kappeler, Saillour et al. 2006, Martini, Valiente et al. 2009). Third, the nucleokinesis occurs, where the nucleus translocate into the leading process (Ayala, Shu et al. 2007, Martini, Valiente et al. 2009). Then interneurons extend the leading process, restarting the cycle.

Under control in vitro conditions, interneurons do not have external molecules that guide them during their migration. Therefore, such interneurons might spend more time exploring their environment and elongate their branches, searching for cues in order to decide where to move. When interneurons are exposed to increasing gradients of repellent cues, in this case Sema3A, they do not need to extend their branches as much as under control conditions. Thus, they might decide where to move in less time. Therefore, interneurons encountering increasing Sema3A gradients might decide which branch will turn into the leading process faster than interneurons under control conditions. If interneurons exposed to Sema3A reduced their exploratory behavior and performed some movement steps in less time, more short processes could be visible than under control condition, as observed in the present results.

It is worth mentioning that interneurons derived from the VZ/SVZ of the MGE migrated away from decreasing gradients of Sema3A and presented processes with similar length than interneurons under control conditions. Time lapse experiments with cocultures could bring further information and clarify the reasons and implications of the morphological changes seen after exposure to increasing gradients of Sema3A, but not when exposed to decreasing Sema3A gradients.

4.2 Sema3C is an attractive guidance molecule for a subpopulation of MGE-derived interneurons during tangential migration.

The present work aimed to elucidate: What is the role, if any, of Sema3C in regulating cortical interneuron migration? Sema3C would be an attractive cue for migrating cortical interneurons as has been already described for extending cortical axons?

First, binding sites for Sema3C on MGE-derived neurons were confirmed. Recombinant proteins tagged with alkaline phosphatase (AP) or a fragment crystallizable (Fc) are useful tools for elucidating binding partners and testing the chemotactic properties of the tagged proteins. Thus, these proteins have been used by others before to describe the binding of Class III Semaphorins (Castellani, Chédotal et al. 2000). In the present study, double immunostaining revealed Nrp1/Sema3C-AP co-localization on MGE-derived interneurons, meaning that these interneurons are bearing Nrp1 receptors and that the recombinant Sema3C protein AP-tagged is able to interact with the Nrp1 receptors present on MGE-derived interneurons.

Next, stripe assays were performed. After using recombinant Sema3C-Fc protein intercalated with control stripes, MGE-derived interneurons were attracted by Sema3C-Fc lanes. This result is consistent with the attractive effects described for membrane bound Sema3C on growing cortical axons (Bagnard, Lohrum et al. 1998, Bagnard, Thomasset et al. 2000). When performing the stripe assay, Sema3C-Fc protein was bound to lanes, distributed homogeneously. However, as mentioned in the previous section, Class III Semaphorins are diffusible molecules that form concentration gradients in vivo (Bagnard, Thomasset et al. 2000, Kruger, Aurandt et al. 2005). Studies have shown that Sema3 gradients are an important feature for modulating the attractive effects on extending cortical axons. Specifically, increasing concentrations of Sema3C attracted and raised the growth speed of cortical axons (Bagnard, Lohrum et al. 1998, Bagnard, Thomasset et al. 2000).

To study whether Sema3C gradients affects MGE-derived cortical interneurons, chemotactic assays were performed, including the Boyden chamber assay and Coculture. The Boyden chamber assays revealed that Sema3C gradients are attractive for MGE-derived interneurons in comparison to control conditions. Therefore, the attractive effect of Sema3C observed with the stripe assay was confirmed in the Boyden chamber experiments and correlates with

previous in vitro assays on extending cortical axons (Bagnard, Lohrum et al. 1998, Bagnard, Thomasset et al. 2000, Ruediger, Zimmer et al. 2013).

Furthermore, in the present study coculture experiments were performed using two types of embryonic tissue. One tissue was the VZ/SVZ of the MGE, which contains neuronal populations that express Nrp1 receptors. The second tissue was the IMZ of the MGE that includes interneurons bearing Nrp2 receptors. (Marin and Rubenstein 2001, Marin, Yaron et al. 2001, Nobrega-Pereira, Kessaris et al. 2008, Zimmer, Rudolph et al. 2011)

Results from coculture experiments illustrate that only interneurons deriving from the VZ/SVZ, but not from the IMZ of the MGE were sensitive to Sema3C gradient. This suggests that most interneurons located in the IMZ of the MGE do not respond to Sema3C. Therefore it is likely that interneurons migrating through the IMZ either lack potential receptor complexes for Sema3C binding, or that Sema3C binding does not affect their migration. Furthermore, the attractive effect of Sema3C on interneurons derived from the VZ/SVZ of the MGE occurred only when exposed to increasing Sema3C concentrations.

The Sema3C attractive effect observed on interneurons derived from the VZ/SVZ of the MGE is analogous with previous results on cortical axons, where increasing concentration of Sema3C attracted them. However, the results obtained whit decreasing concentrations of Sema3C are different from elongating axons and migrating interneurons. Interneurons derived from the VZ/SVZ of the MGE were not affected by decreasing concentrations of Sema3C, while previous reports have shown that decreasing concentrations of Sema3C affect cortical axons by reducing their growth speed (Bagnard, Lohrum et al. 1998, Bagnard, Thomasset et al. 2000).

Sema3C binding to Nrp-Plexin receptor complexes activates intracellular pathways that seems to regulate Rho family proteins and thus control actin dynamics. Such actin dynamics mediated changes in the shape or trajectory of the interneurons bind to Sema3C (Castellani and Rougon 2002, Hatanaka, Matsumoto et al. 2009, Niquille, Garel et al. 2009, Piper, Plachez et al. 2009, Ruediger, Zimmer et al. 2013). Therefore, the present study examined if any changes occurred in the morphology of MGE-derived interneurons exposed to Sema3C. Using the collapse assays and phalloidin staining, growth cones only stretched more than under control conditions. Regarding the area of the growth cones, no significant differences in the growth cone of the leading process were observed after Sema3C exposure or control conditions. This result was similar to previous observations on growth cones of corticofugal

axons exposed to Sema3C (Bagnard et al., 1998; Bagnard et al 2000 (Bagnard, Lohrum et al. 1998, Bagnard, Thomasset et al. 2000). Thus, corticofugal axons and MGE-derived interneurons are both attracted by Sema3C and their growth cones seems to respond on similar ways, remaining basically unchanged when encountering Sema3C.

Using coculture assays, no significant differences in the length of the leading processes were observed between Sema3C gradients and control conditions. As discussed in the previous section, the interneurons reduce their processes only when encounter an increasing concentration of Sema3A repellent cue. Interneurons derived from the VZ/SVZ of the MGE were stimulated to move towards the Sema3C increasing gradient. Also, such interneurons moved away from Sema3A decreasing gradient. In both cases, the length of the leading neurite remains similar than under control conditions. Therefore, interneurons exposed to decreasing gradients of Sema3C or Sema3A extend their processes, screening the molecules in their environment as much as under control conditions. As already mentioned, further experiments, like time lapse with Coculture, could elucidate the effects of gradients of guidance cues on interneuron morphology and their implication in tangential migration.

So far, one long and short range molecule described as attractant for a subpopulation of migrating MGE-derived interneurons is Neuroregulin 1 (Nrg1). Nrg1 signals through ErbB4 receptors. Membrane bound Nrg1-CRD (Cysteine Rich Domain) creates a permissive corridor that channel ErbB4 positive MGE-derived interneurons through the lateral ganglionic eminence (LGE), helping to avoid the striatum. On the other hand secreted Nrg1-Ig (Immunoglobulin-like domain) isoform strongly attracts a subpopulation of MGE-derived interneurons that bear the ErBb4 receptor towards the neocortex (Flames, Long et al. 2004). Since ErbB4 is only expressed by a subpopulation of interneurons, the existence of other long range attractive molecules, like Sema3C, that might guide subpopulation of cortical interneurons towards the neocortex, seems likely.

Extrapolating the present *in vitro* result to *in vivo* conditions, it seems possible that the area of the neocortex, where Sema3C is highly expressed, creates a permissive zone for cortical interneurons. Such permissive area might allow migrating cortical interneurons to pass the pallial/subpallial border and stimulates them to enter into the intercortical subventricular zone of the neocortex. Such attractive effects could be simultaneous, additive or sequential to the already described Nrg1 effects. Also, Cxcl12 is a diffusible protein modulated by CxCR7 receptors and likely restrained by heparan sulfates (Reiss, Mentlein et al. 2002). Cxcl12

creates attractive corridors to guide CxCR4-positive MGE-derived interneurons in the neocortex and seems to control their sorting and laminar positioning on the cortical layers (Li, Adesnik et al. 2008, Marín, Valiente et al. 2010, Tanaka, Mikami et al. 2010, Lysko, Putt et al. 2011, Sanchez-Alcaniz, Haege et al. 2011, Wang, Li et al. 2011, Marin 2013). Such corridors are in the marginal zone and the subventricular zone of the neocortex (Tham, Lazarini et al. 2001, Marín, Valiente et al. 2010, Sanchez-Alcaniz, Haege et al. 2011, Marin 2013). The similarity in the attractive role and expression patterns of Sema3C and Cxl12, raises the possibility that Sema3C might also play a role in confining migrating interneurons into specific routes towards the neocortex.

Another aspect that the present study prompts to investigate is: how do migrating cortical interneurons respond to opposing cues like Sema3A and Sema3C? In the basal telencephalon, gradients of Sema3A and Sema3C might temporally and spatially overlap. On migrating interneurons derived from the ventricular and subventricular zone of the MGE, Sema3A signals through Npr1 receptors (Marin, Yaron et al. 2001, Zimmer, Schanuel et al. 2010). In the present study, interneurons derived from the VZ/SVZ of the MGE also showed Sema3C-Nrp1 co-localization. Thus, migrating cortical interneurons bearing Nrp1 receptors might need to integrate Sema3A and Sema3C opposing cues.

Previous reports have described the integration of Sema3A and Sema3C opposing cues on cortical axons. Sema3C and Sema3A gradients were shown to overlap in the dorsal telencephalon and delineate the early path of corticofugal projections (Ruediger, Zimmer et al. 2013). Thus, efferent cortical axons were driven deep into the intermediate zone of the neocortex by Sema3C attraction. Next, Sema3A repulsion overrides Sema3C attraction on cortical axons. Therefore cortical axons avoided the area were Sema3C and Sema3A overlap and growth in the region were only Sem3C is expressed (Ruediger, Zimmer et al. 2013).

When Sema3C and Sema3A overlap only disulfide linked homodimers are formed and no Sema3A/Sema3C heterodimer ligand complexes (Adams, Lohrum et al. 1997, Koppel and Raper 1998). In contrast, Neuropilin proteins, Nrp1 and Nrp2, are membrane bound receptors that can form both homodimers and heterodimers. Binding studies revealed a high affinity of Sema3A to Nrp1, but not to Nrp2. In contrast, Sema3C binds with a high affinity to both Nrp1 and Nrp2 receptors (Chen, Chedotal et al. 1997, He and Tessier-Lavigne 1997, Kolodkin, Levengood et al. 1997, Takahashi, Fournier et al. 1999, Gu, Rodriguez et al. 2003). Sema3C interactions on cortical axons rely on Nrp1/Nrp2 heterodimers whereas Sema3A

effects rely on Nrp1 homodimers (Chen, Chedotal et al. 1997, Takahashi, Fournier et al. 1999, Ruediger, Zimmer et al. 2013).

Sema3A is not only expressed in the developing striatum but also in the dorsal telencephalon (Bagnard, Lohrum et al. 1998, Marin, Yaron et al. 2001, Zimmer, Schanuel et al. 2010). Such Sema3A expression repels extending cortical axons in the dorsal telencephalon and migrating interneurons in the basal telencephalon (Bagnard, Lohrum et al. 1998, Marin, Yaron et al. 2001, Zimmer, Schanuel et al. 2010). Moreover, Sema3A expressed in the dorsal telencephalon, repels migrating cortical interneurons that reached the neocortex (Tamamaki, Fujimori et al. 2003). Sema3A and Sema3C opposing cues, which overlaps in the dorsal telencephalon, might play a role in the intracortical migration of interneurons, confining them through the SVZ and the MZ of the neocortex.

An interesting aspect of the present study is that only interneurons from the deep migratory stream are attracted by Sema3C through Nrp1 receptors. This raises the question, how migrating interneurons following the deep migratory stream differentiate between Sema3A and Sema3C responses. Further studies should provide some insights in the cellular mechanisms of neuronal migration in a way that resembles more the in vivo situation. For example, cortical axons seem to modulated Sema3A and Sema3C opposing effects through Nrp2/Nrp1-Sema3C binding (Ruediger, Zimmer et al. 2013). In migrating cortical interneurons both, Sema3A repulsion and Sema3C attraction, signal through Nrp1 receptor. Since Neuropilin receptors are segregated in the deep and upper migratory stream, migrating cortical interneurons cannot form Nrp1/Nrp2 to modulate Sema3s opposing effects.

Since Neuropilins have a short cytoplasmatic domain, they cannot activate an intracellular signaling cascade themselves. Thus, Neuropilin needs to associate with Plexin proteins and form receptor complexes (Takahashi, Fournier et al. 1999, Kruger, Aurandt et al. 2005). Therefore, one possible explanation for Sema3A and Sema3C signaling through Nrp1 on cortical Interneurons is that Sema3A-Nrp1 has more affinity to a different Plexin than Sema3C-Nrp1. *In vitro* studies have shown that PlexinA1 and PlexinD1 form complexes with Nrp1 or Nrp2 to bind Sema3A, Sema3F or Sema3C (Gitler, Lu et al. 2004, Pasterkamp 2012). So far, PlexinA1 is the generally accepted co-receptor for Sema3A, Sema3F or Sema3C (Takahashi, Fournier et al. 1999, Pasterkamp 2012), but further studies are needed to

confirm which Plexin mediates Sema3A, Sema3C or Sema3F signaling on migrating cortical interneurons.

There is also the possibility that specific Class III Semaphorin binding desensitized migrating interneurons to others guidance molecules, including others Sema3s. What have been shown is that intrinsic Sema3A or Sema3C can modulate Nrps levels and thereby further responsiveness to Nrp1 and Nrp2 dependent Sema3 proteins (Moret, Renaudot et al. 2007, Sanyas, Bozon et al. 2012). For example, motor axons innervating the forelimb muscles are exposed to combined expressions of class III Semaphorins. Endogenous Sema3C on motoneurons upregulates growth cones responsiveness to exogenous Sema3F, whereas desensitized them to Sema3A (Sanyas, Bozon et al. 2012).

Sema3C attractive effects through Nrp1 could also be modulated by other co-receptors. Such possibilities have already been described for Robo1-Nrp1 or Limk2-PlexinA1 that indirectly modulate Sema3A repulsion on migrating MGE-derived cortical interneurons (Hernández-Miranda 2011, Andrews, Zito et al. 2013). Ig superfamily cell adhesion molecules (IgSFCAMs) such as L1, have also been found to confer a specific response to Sema3A (Castellani, Chédotal et al. 2000, Castellani and Rougon 2002, Castellani, Falk et al. 2004, Wright, Demyanenko et al. 2007, Hernández-Miranda 2011) Studies have suggested that L1 interact with Nrp1 receptors and L1/Sema3A interaction repels cortical axons in the spinal cord (Castellani, Chédotal et al. 2000, Castellani, Falk et al. 2004)).

In conclusion, the present study confirms the repellent effect suggested for increasing concentrations of Sema3A. Additionally, it was shown that decreasing concentrations of Sema3A promoted the migration of cortical interneurons derived from the VZ/SVZ of the MGE. Furthermore, cortical interneurons follow the increasing gradient of Sema3C towards the cortex. It is likely that such cortical interneurons following Sema3C attraction bear Nrp1 as part of their receptor complex. As summarizes the Figure 24, these *in vitro* results could implicate that *in vivo*, cortical interneurons derived from the ventricular/subventricular zone of the MGE, do not only avoid the striatum by Sema3A repulsion, but also seemed to be stimulated to keep on moving away from the Sema3A decreasing gradient towards the cortex. Finally, cortical interneurons encountering the increasing gradient of Sema3C seems to be attracted by this cue towards the neocortex.

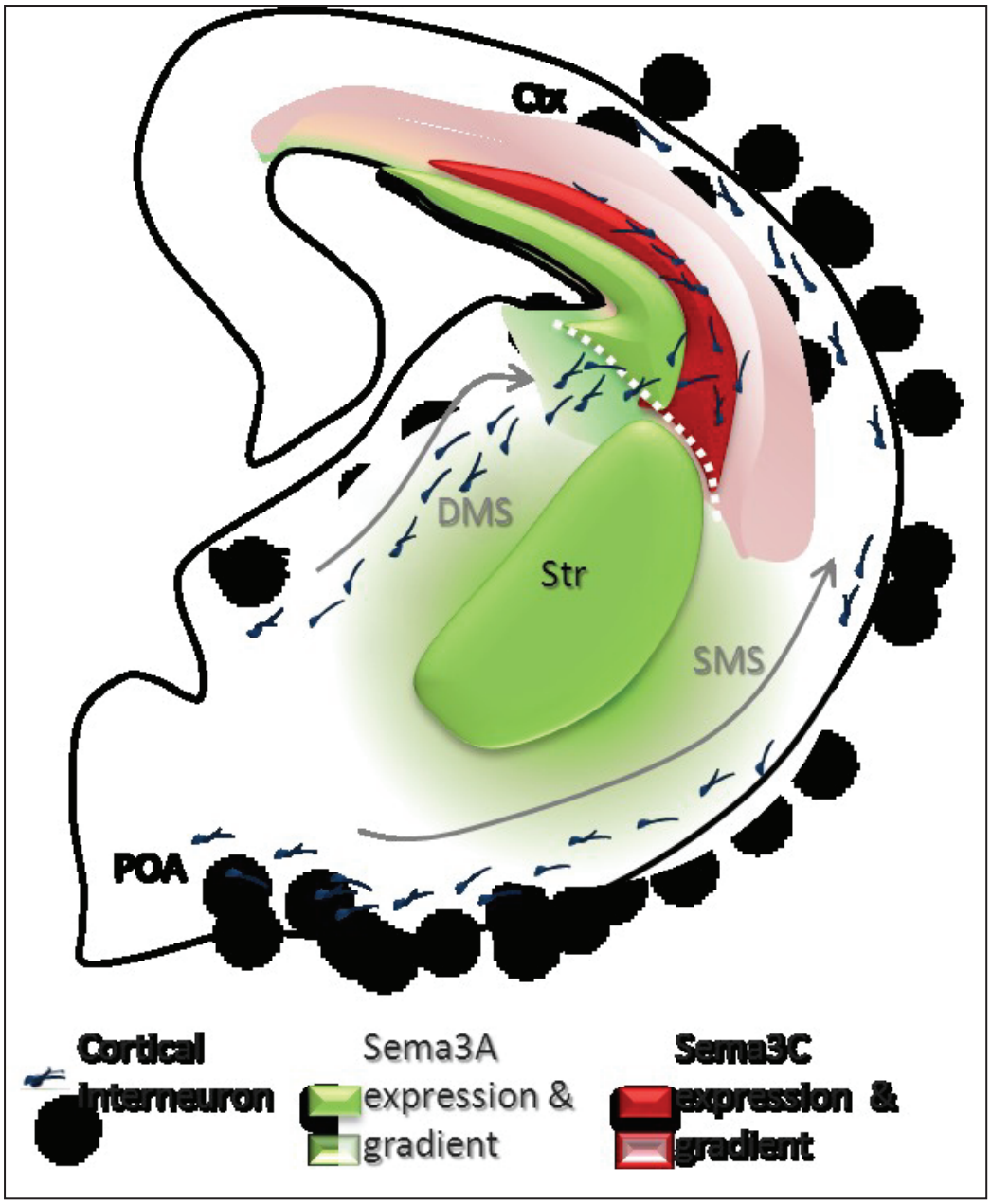


Figure 24. Sema3C and Sema3A gradients channel migrating MGE-derive interneurons towards the neocortex. Illustration of an E14.5 coronal brain slice with the expression and possible distribution patterns of Sema3A and Sema3C. Sema3A and Sema3C are secreted, diffusing and thereby creating gradients. Sema3A prevents cortical interneurons in the superficial and deep migratory stream from entering the striatum. Also, Sema3A decreasing gradients keep cortical interneurons moving away from the repellent source on the DMS. Next, the increasing Sema3C gradient attracts the interneurons on the DMS promoting the crossing of the pallial/subpallial border –white dotted line- towards the intermediate and subventricular zone of the neocortex. In the dorsal telencephalon, Sema3A is also express in the VZ and repels migrating interneurons (Tamamaki et al., 2003). In contrast, Sema3C seems to create a permissive corridor and might attract interneurons towards the subventricular and intermediate zone, into the intracortical pathway. How cortical interneurons integrates Sema3A and Sema3C opposing guidance cues during their migration, remains to be elucidated. Ctx, Cortex; Str, Striatum; DMS, Deep Migratory Stream; SMS, Superficial Migratory Stream; MGE, Medial ganglionic eminences; POA, Preoptic area.

5. Summary

During embryonic brain development, cortical interneurons are born in the basal telencephalon and migrate tangentially to the neocortex. Thereby, interneurons derived from the ventricular and subventricular zone (VZ/SVZ) of the medial ganglionic eminences (MGE) mostly follow precise pathways along the deep migratory stream, whereas interneurons derived from the preoptic area (POA) mainly migrated over the superficial migratory stream passing through the intermediated zone (IMZ) of the MGE. These migratory routes are regulated by several guidance cues, including Semaphorins, along the path or in flanking regions. While it is known that Semaphorins act as guidance cues for growing axons during brain development (Bagnard, Lohrum et al. 1998, Ruediger, Zimmer et al. 2013), their potential role during interneuron migration is largely unknown. It has been shown that Sema3A is expressed in the developing striatum and acts as a repulsive cue for migrating cortical interneurons, preventing them from innervating this non-target areas (Marin et al., 2001(Marin, Yaron et al. 2001)). One striking observation is that Sema3C demarcates the pallial/subpallial border and the intracortical pathway of cortical interneurons in the dorsal telencephalon (Bagnard, Lohrum et al. 1998, Ruediger, Zimmer et al. 2013). Furthermore, Sema3s are secreted proteins, therefore Sema3C might be able to form a gradient that reach cortical interneurons earlier on their route from the basal telencephalon towards the neocortex. Moreover, migrating cortical interneurons express Nrp1 and Nrp2, described receptors for Sema3A and Sema3C. All these reasons prompt to examine possible roles for Sema3C on cortical interneuron migration.

Here, several in vitro assays were performed to examine the role of Sema3C and Sema3A gradients on tangential migrating interneurons. The stripe assay pointed out that MGE-derived interneurons grow preferentially on Sema3C stripes, compared to control stripes, indicating an attractive effect on cortical interneurons. Results from coculture assays confirm the repulsive effects of Sema3A on MGE-derived interneurons from the ventricular and subventricular zone. A novel observation from the present study is that decreasing Sema3A gradients stimulated interneurons from VZ/SVZ explants out of the MGE to move longer distances away from the Sema3A source. Moreover, Sema3A acts as a repellent cue for interneurons from the intermediate zone (IMZ) of the MGE. In contrast, only Sema3C increasing gradients attracted MGE-derived neurons specifically from the VZ/SVZ and not from the IMZ, whereas decreasing Sema3C gradients seems to have no effect. Furthermore,

the morphological changes of cortical interneurons elicited by Sema3A or Sema3C were studied in detail. Results indicated that MGE-derived interneurons from the VZ/SVZ exposed to Sema3C only presented a stretchment in their growth cone without any change in its total area. In contrast, interneurons encountering Sema3A, exhibit an enlargement of their growth cone and shorter neurites. This suggests that Sema3A might trigger the regulation of the cytoskeleton.

In summary, the experiments indicated that Sema3C is an attractive cue for cortical interneurons in the deep migratory stream. These in vitro results could implicate that in vivo, cortical interneurons derived from the subventricular and ventricular zone of the MGE, do not only avoid the striatum by Sema3A but also seemed to be stimulated to keep on moving away from the Sema3A decreasing gradient towards the cortex. Finally, cortical interneurons encountering the increasing gradient of Sema3C seem to be attracted by this cue towards the neocortex.

6 Zusammenfassung

Während der embryonalen Gehirnentwicklung werden kortikale Interneurone im basalen Telenzephalon geboren und migrieren dann tangential in den Neokortex ein. Interneurone aus der Ventrikularzone und Subventrikularzone (VZ/SVZ) der medialen ganglionischen Eminenz (MGE) folgen dabei hauptsächlich dem tiefen Migrationsstrom (DMS), während Interneurone des präoptischen Areals (POA) hauptsächlich im superfiziellen Migrationsstrom (SMS) durch die Intermediärzone (IMZ) der MGE migrieren. Die Migration der kortikalen Interneurone wird durch zahlreiche Lenkungsfaktoren gesteuert, die entlang ihres Weges oder von flankierenden Regionen exprimiert werden. Auch Semaphorine stellen solche potentiellen Lenkungsmoleküle dar. Während bereits bekannt ist, dass Semaphorine während der Gehirnentwicklung auswachsende Axone leiten (Bagnard, Lohrum et al. 1998, Ruediger, Zimmer et al. 2013), ist wenig über ihre mögliche Rolle während der Migration kortikaler Interneurone bekannt. Sema3A wird im sich entwickelnden Striatum exprimiert und wirkt repulsiv auf migrierende kortikale Interneurone, welche davon abgehalten werden in das Striatum einzuwandern (Marin, Yaron et al. 2001). Interessanterweise wird Sema3C an der pallialen/subpallialen Grenze und entlang des intrakortikalen Migrationsweges der kortikalen Interneurone im dorsalen Telenzephalon exprimiert (Bagnard, Lohrum et al. 1998, Ruediger, Zimmer et al. 2013). Da es sich bei Klasse 3 Semaphorinen um sezernierte Proteine handelt, liegt Sema3C in Form eines Gradienten vor. Dieser dient erreicht die kortikalen Interneurone bereits im basalen Telenzephalon und leitet sie auf ihrem Weg in den Neokortex. Kortikale Interneurone weisen die Rezeptoren Nrp1 und Nrp2 auf, welche potentielle Bindungspartner von Sema3A und Sema3C darstellen. All diese Befunde deuten auf eine mögliche regulatorische Rolle von Sema3C auf die Migration kortikaler Interneurone hin.

Um einen möglichen Einfluss von Proteingradienten der Signalmoleküle Sema3C und Sema3A auf die tangentiale Migration kortikaler Interneurone zu untersuchen, wurden zahlreiche in vitro Experimente durchgeführt. Dabei zeigte der Streifen-Assay zunächst, dass Interneurone aus der MGE bevorzugt auf Sema3C Streifen wachsen. Das deutet darauf hin, dass Sema3C ein attraktives Signal für kortikale Interneurone darstellt. Die repulsive Wirkung von Sema3A auf Interneurone aus der VZ/SVZ der MGE konnte im Rahmen von Cokultur-Experimentes bestätigt werden. Darüber hinaus wirkt Sema3A auch repulsiv auf Interneurone aus der Intermediärzone (IMZ) der MGE. Eine interessante Beobachtung dieser

Arbeit war, dass sowohl ein ansteigender, als auch ein absteigender Sema3A Gradient repulsiv auf Interneurone aus MGE -Explantaten der VZ/SVZ wirkt. Das Signalmolekül stimuliert die Interneurone, weitere Strecken weg von der Sema3A-Quelle zu migrieren. Im Gegensatz dazu wirken nur ansteigende Sema3C Gradienten attraktiv auf Interneurone aus der VZ/SVZ und aus der IMZ der MGE. Während absteigende Sema3C Gradienten diesen Effekt nicht ausüben. Weiterhin wurde auch erstmals untersucht, dass Sema3A und Sema3C morphologische Unterschiede in kortikalen Interneuronen induzieren. Die Ergebnisse deuten darauf hin, dass Interneurone von der VZ/SVZ der MGE eine vergrößerten Wachstumskegel und kürzere Neuriten aufweisen, wenn sie mit Sema3A in Kontakt kommen. Diese Beobachtungen weisen darauf hin, dass Sema3A in die Regulation des Zytoskeletts involviert ist.

Die Daten dieser Arbeit deuten darauf hin, dass Sema3C attraktiv auf kortikale Interneurone entlang des tiefen Migrationsstromes wirkt. Die in Vitro Resultate weisen darauf hin, dass Sema3A die kortikalen Interneurone aus der VZ/SVZ der MGE davon abhält, in das sich entwickelnde Striatum einzuwandern. Darüber hinaus scheint der absteigende Gradient des Signalmoleküls die Interneurone zu stimulieren, die Migration vom basalen Telenzephalon in der Neokortex fortzusetzen. Im Gegensatz dazu scheint der ansteigende Sema3C Gradient auf dem Weg der kortikalen Interneurone diese hin zum Neokortex leiten.

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Chapter 8: Annex

8.1. Abbreviations

µl	Microliter
µm	Micrometer
µm2	Square micrometer
µM	Micro molar
AP	Alkaline phosphatase
Bidest	Double-distilled water
CRD	Cysteine rich domain
Ctx	Cortex
DAPI	4 ',6- diamidino -2-phenylindole
CXCL12	C-X-C motif chemokine 12 also known as SDF-1
CXCR4	C-X-C chemokine receptor type 4
CXCR-7	C-X-C chemokine receptor type 7
DMEM	Dulbecco 's Modified Eagle 's Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethyldiamintetraessigsäure
FBS	Fetal bovine serum
ErbB4	Receptor tyrosine-protein kinase
Fc	Fragment crystallizable (Fc)
GBSS	Gey 's balanced salt solution
h	Hour
HBSS	Hanks'balanced salt solution
HEK	Human Embryonic Kidney cells
HNO3	Nitric acid
H2O2	Hydrogen peroxide
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
IMZ	Intermediate zone

kDa	Kilo Dalton
L1	Neural Cell Adhesion Molecule
LGE	Lateral ganglionic eminence
LIMK2	LIM domain kinase 2
M	Molar
min	Minutes
MGE	Medial ganglionic eminence
mM	Millimolar
MEM	Modified Eagle 's Medium
mU	Milli units
n	Sample size
Nrg1	Neuroregulin 1
Nrp1	Neuropilin 1
Nrp2	Neuropilin 2
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PLAP	Placental Alkaline Phosphatase
POA	Preoptic area
rpm	Rotations per minute
RT	Room temperature
SD	Standard Deviation
SDF-1	stromal cell-derived factor 1
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Str	Striatum
SVZ	Subventricular
Tris	Tris (hydroxymethyl) aminomethane
Triton-X-100	Octylphenoxypolyethoxyethanol
U	Units
VZ/SVZ	Ventricular zone/Subventricular zone

8.2 Reagents

8.2.1. Buffer solutions and reagents

For in vitro experiments only sterile buffers and solutions were used. Buffers and solutions which sterility were not guaranteed by the manufacturer, were either sterilized using a sterile filter pore (size of 0.2 micron Sartolab V 115; SARTORIUS bar) or autoclaved for 20 min at 121 ° C. When indicated bi-distilled (Bidest) water was used as solvent.

8.2.1.1. Anesthetic used for culled the mice

Chloral hydrate: Storage at room temperature	10% Chloral hydrate in PBS

8.2.1.2. Primary culture

PBS:	120 mM NaCl
Storage at 4 °C	2.7 mM KCl
	10 mM Na2HPO4
	pH = 7,4
GBSS:	1.53 mM CaCl2
Storage at 4 °C	3.66 mM KCl
	0.22 mM KH2PO4
	1.03 mM MgCl2-6H2O
	0.28 mM MgSO4-7H2O
	138 mM NaCl
	2.70 mM NaHCO3
	0.84 mM Na2HPO4
	5.56 mM Glucose
65 % Glucose solution	32.5 g D-(+)-Glucose
(m/v):	in 50 ml Bidest
Storage at -20 °C	

0,25 % Trypsin (v/v):	Trypsin at 25 %
Storage at -20 °C	1:10 in PBS
Preparation medium:	0.65 % Glucose
Freshly prepared before used	in GBSS
Collection medium:	0.65 % Glucose
Freshly prepared before use	in HBSS without magnesium and calcium
Culture medium for single cells (with FBS):	10 % heat-inactivated Fetal Bovine Serum (FBS)
Storage for max. 7 days at 4 ° C	1 % Penicillin/Streptomycin
	200 mM L-Glutamine
	0,1 % D-Glucose
	in DMEM with Natriumpyrovat and Pyridoxin
Neurobasal medium:	0,5 % D-Glucose
	1 % L-Glutamine
	1 % Penicillin/Streptomycin
	2 % B27 Supplement
	1 % ml N2 Supplement
	In Neurobasal
Laminin-poly-L-lysine Mixture:	19.5 µl laminin solution (1 mg/ml)
	5 µl poly-L-lysine solution (10 mg/ml)
	in sterile GBSS.

8.2.1.3 Preparation of glass coverslips

Degreased the rectangular coverslips (12 x 14 mm) or round coverslips (diameter: 9 mm) by heating in absolute alcohol at 60 °C for 30 min, and sterilized them (after evaporation of the

alcohol) for three hours at 140 °C. Also, the coverslips used on the stripe assay and coculture, were cleaned using HNO3.

The coating of the cover glass was carried out with 100 µl of fresh laminin-poly-L-lysine mixture in each experiment.

Apply 100ml mixture per cover slip “sandwich”. Cover with a second coverslips. Incubate (37°C, 5 % CO2) for at least 30 min. Separate the “sandwiches”, rinse them in Bidest water and let them dry. All the procedures of coating are perform under sterile conditions (in the laminar flow bench) and the covered part of the coverslip should be facing upwards to maintain the protein mixture.

8.2.1.4. Solutions and media for cell culture: HEK cells

Cell culture medium:	10 % heat-inactivated FBS
Storage at 4°C	1 % Penicillin/Streptomycin
	in MEM
	only for transfected HEK cells (not in the first passage or
	after thawing):
	0.5 % Geneticin
Freezing medium:	90 % Cell culture medium
	10 % DMSO

8.2.1.5. Fixation and immunohistochemistry

Fixation solution:	4 % PFA
Storage at -20°C	Dissolve in PBS at 60°C
	pH=7,4;
Washing buffer:	0,2 % Triton-X-100
Storage at 4°C	in PBS

Blocking solution: 1 % BSA
Storage at 4°C 0,2 % Triton-X-100
in PBS

Mowiol: 25 % Moviol (m/v)
Storage at -20°C 50 % Glycerin (v/v)
17,5 % n-Propyl-Gallat (m/v)
0,03 % Timerosal
in PBS

8.2.1.6 Photometric quantification of Semaphorin-AP activity

AP substrate solution: 2 mg/ml 4-nitrophenyl phosphate
100 mM NaCl
20 mM MgCl2
in 100 mM Tris-HCl
pH 9.5

8.2.1.7. SDS-Gel electrophoresis and Western Blot

8.2.1.7.1 Performed as detail in section 2.5.1

STEN Buffer: 50 mM Tris HCL, pH 7.6
150 mM NaCl
2 mM EDTA
1 % NP-40
1: 500 Protein Inhibitor Mix, add fresh

Laemmli sample buffer (4 X): 5 ml of 0.5 M Tris HCl, pH = 6.8
4 ml of 20 % SDS
4 ml Glycerol

	1 ml ß-Mercaptoethano add fresh to 9 ml stock
Sample mix:	20 µl Sample from HEK Cells.
	4 µl 6 X Laemmli Sample Buffer
SDS running buffer	0,25 M Tris
(10 x):	14,4 % Glycine (m/v)
	1% SDS
SDS (10 X):	60.4 g Tris base
-To add in the SDS	288 g Glycine
running buffer-	to 2 l with water
0.25 M Tris	pH ~6.8, check pH but NEVER adjust. It should be
14.4 % Glycine (w/v)	automatically pH ~6.8
Blot Buffer (10 X):	0.25 M Tris
	14.4 % Glycine (w/v)

8.2.1.7.1 1 For preparing the 8 % Acrylamide Gel

Upper Tris (4 X):	30.3 g Tris base
0.5 M Tris	10 ml 20 % SDS
0.4 % SDS	to 500 ml bidest
	pH 6.8 adjust with HCL
Lower Tris (4 X):	181.7 g Tris base
1.5 M Tris	20 ml 20 % SDS
0.4 % SDS	to 1 l Bidest
	pH 8.8 adjust with HCl
Separating Gel:	3,87 ml H2O
	2.13 ml AA-Mix (30%)
	2,00 ml Lower Tris (4x)

15 µl TEMED APPLY right before use!

15 µl 10% APS

Stacking Gel: 3,15 ml H2O

0.60 ml AA-Mix (30%)

1.25 ml Lower Tris (4x)

15 µl TEMED APPLY right before use!

15 µl 10% APS

8.2.1.7.2 SDS-Gel electrophoresis, performed as described in section 2.5.3

STEN Buffer: 50 mM Tris-HCL pH 7.6

150 mM NaCl

2 mM EDTA

1 % TritonX-100

1: 100 PI-Mix, add fresh

Sample mix: 10 µl Sample from HEK cells

2,4 µl 10 X reducing agent

6 µl 4 X Laemmlin Buffer

5.6 Bidest

Running Buffer: 700 ml MOPS (20 X)

200 ml are for the outer compartment

500 ml with 500 µl antioxidant are for the inner compartment

Blotting Buffer: 25 mM Tris

Storage at room temperature 192 mM Glycine

20 % Methanol

0.02 % SDS

pH between 8.2 and 8.4 NEVER adjust.

TBS-T:	200 mM Tris
TBS-T (10 X)	1,5 M NaCl
	1 % Tween 20
	pH 7,5
Blocking solution:	5 % Milk powder
	on TBS-T

8.2.1.7.2.1 For development with ECL Method

DAB Buffer	4.5 ml Tris-HCL (50 mM)
	10 µl H2O2
	0.5 ml DAB

8.2.1.7.3 Ladders loaded in the SDS-Blot

Type	**Manufacture**
ECL protein molecular weight markers	AMERSHAM
Store at -20 ° C	
SDS molecular weight markers	SIGMA
Store at -20 ° C	
Spectra Multicolor High Range	SPECTRA
Store at -20 ° C	

8.2.2 Recombinant protein and antibodies

8.2.2.1 Recombinant protein

Type	**Manufacture**	**Concentration**
Recombinat Human Semaphorin 3C-Fc chimera	R&D	50 mg/µl

8.2.2.2. Antibodies

8.2.2.2.1 Primary antibodies

Type	Manufacturer	Specificity	Final dilution
Anti Semaphorin 3C	R&D	Sheep IgG	1:1000
Anti Semaphorin 3A	R&D	Goat IgG	1:1000
Anti PLAP	ABD-SEROTEC	Rabbit	1:100
Anti Nrp1	R&D	Goat IgG	1:1000

8.2.2.2.2 Secondary antibodies and dyes

Type	Manufacture	Fluorescent conjugate	Final dilution
Anti-human	INVITROGEN	Alexa 488	30 µg/ml
Donkey anti goat	JACKSON	Cy3	1:1000
Donkey anti rabbit	MOLECULAR	Alexa 488	1:1000
Phalloidin	BIOTIUM	Rodamine ---	1:100

8.2.2.2.2.1 Used for detecting the bands in the SDS-Gel electrophoresis, section 2.5.1

Type	Final dilution
Anti sheep Horseradish Peroxidase (HRP)	1:1000
Anti goat Horseradish Peroxidase (HRP)	1:2000

8.2.2.2.2.2 Used for detecting the bands in the SDS-Gel electrophoresis, section 2.5.3

Type	Manufacture	Final dilution
Biotinyl rabbit anti sheep	VECTOR	1:1000
Biotinyl rabbit anti goat	VECTOR	1:1000

8.2.3 Chemicals manufacture

Product	Manufacture
4-12 % Bis-Tris Gradient gel	INVITROGEN

4-nitrophenyl phosphate (for AP substrate)	SIGMA
ABC reagent	VECTSTATIN
B2	LIFE TECHNOLOGIES
CaCl2	SIGMA
Chicken plasma	SIGMA
Chloral hydrate	SIGMA
Chloroform	MERCK
DAPI	SIGMA
DMEM	INVITROGEN
DMSO	INVITROGEN
ECL	AMERSHAM
EDTA	SIGMA
EtOH	MERCK
Fc	ROCKLAND INC.
Fixating salts	SIGMA
Fetal bovine serum	INVITROGEN
Geneticin G 418 strain	INVITROGEN
Glucose	SIGMA
Glycerol	SIGMA
HBSS	INVITROGEN
Image Quant, Las 4000 mini	FUJI
Isopropyl alcohol	SIGMA
KCl	MERCK
KH2PO4	MERCK
Laminin	SIGMA
MEM	INVITROGEN
Methanol	ROTH
MgCl2	MERCK
MgCl2 6 H2O	MERCK
MgSO4 7 H2O	MERCK
Mineral oil (For 100 X oil objective)	SIGMA
MOPS running buffer 20 X	INVITROGEN

Mowiol4 -88	CALBIOCHEM
N2	LIFE TECHNOLOGIES
Na2HPO4	SIGMA
Na3VO4	SIGMA
NaCl	ROTH
NaF	SIGMA
NaHCO3	SIGMA
NaOH	MERCK
NBT	ROCHE
Neurobasal	GIBCO
n- propyl gallate	SIGMA
Paraformaldehyde	MERCK
Penicillin / streptomycin	INVITROGEN
PepstatinA	SIGMA
Peptone	INVITROGEN
Phenylmethylsulfonyl	SIGMA
Poly-L -lysine	SIGMA
Sample buffer for SDS PAGE	INVITROGEN
Protein inhibitor cocktail mix	SIGMA
Protein marker Multicolor High range	SPECTRA
Reducing Agent	INVITROGEN
SDS, Sodium dodecyl sulfate	ROTH
Timerosal	SIGMA
Tris	SIGMA
Triton X- 100	SIGMA
Trypsin	INVITROGEN
Tween 20	MERCK

8.3 Equipment

Product	**Manufacture**
Axiovert S-100 inverted microscope	ZEISS
Boyden chamber, cell haptotaxis kit	CELL BIOLABS

Electrophoresis Power Supply	GIBCO BRL
Electrophoresis module, mini protein 3 cell	BIORAD
Fastblot B43	BIOMETRA
Filter device (For ≥ 100 kDa)	MILLIPORE
Filter paper (3 mm)	AMERSHAM
Fix rotor Centrifuge F45-30-11	EPPENDORF
Glass Coverslips	MENTZEL
Hot-beat Sterilizer	FINE SCIENCE TOOLS
HERA cell incubator	HERAEUS
Laminar flow hood HERA SAFE	HERAEUS
Micro plate reader, Mithras LB940, 96 wells	BERTHOLD TECHNOLOGIES
Cell counter chamber	NEUBAUER
Nylon net, mesh size: 120 µm	MILLIPORE
Petri Dishes	GREINER BIO_ONE
PVDF Immobilon-P transfer membrane	MILLIPORE
Razor blades	WILKENSON
Spot camera	VISITRON SYSTEMS
Silicone matrices for the Stipe assay	MAX-PLANCK INSTITUTE for Developmental Biology. Tübingen, Germany
TCS SP5 confocal microscope	LEICA
Tempcontrol 37-2 digital	THE MICKLE LABORATORY ENGENEERING
Tissue chopper	SHIMADZU
UV-visible spectrophotometer UV-1601	HERAEUS
Waterbath	INLABO

8.4 Eidesstattliche Erklärung/Declaration of honor

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt habe. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte unmittelbar oder mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Dissertation wurde weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung, noch an einer anderen Hochschule als Dissertation eingereicht. Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät ist mir bekannt. Ich versichere, dass die vorliegende Dissertationsschrift bisher weder im In- noch im Ausland in gleicher oder in wesentlichen Teilen ähnlicher Form als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht oder einer anderen Hochschule zur Eröffnung eines Verfahrens zum Erwerb eines akademischen Grades vorgelegt wurde.

I hereby confirm that I prepared the present academic work and carried out myself the activities directly involved with it. I also confirm that I have followed the current doctoral regulations of the Faculty of Biology and Pharmacy form the FSU, Jena. In the present study I used no resources other than those declared. All formulations and concepts adopted literally or in their essential content from printed, unprinted or Internet sources have been cited according to the rules for academic work. The support provided during the work, including significant assistance from my supervisor has been indicated in full. The academic work has not been submitted to any other examination authority. I am aware that a false declaration will have legal consequences.

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Kiara Aiello

8.5 Curriculum vitae

Kiara Aiello

Date of Birth: 27.November.1986 in Caracas Venezuela
Nationality: Italian and Venezuelan.
Email: aiekia@hotmail.com

Education

2010- Present	**PhD Neurobiology, Friedrich Schiller University (FSU). Jena, Germany**
Institute:	General Zoology and Animal Physiology Institute
Tutor:	Prof. Dr. Jürgen Bolz
Scholarship:	August 2010/June 2013- "! Ánimo, Chévere!", **Erasmus Mundus** exchange program October & November 2013 "PhD competition scholarship", **FSU Graduate Academy**
Theme:	"Sema3C guides MGE-derived cortical interneurons through the basal telencephalon"
Workshops:	Scientific Writing, Academic Teaching, Scientific Presentations and Leadership skills. Offered by the FSU Graduate Academy
2004-2010	**Diploma in Biology, Simon Bolivar University. Caracas, Venezuela** 5 years degree, equivalent to a Master of Biological Science in Europe
Modules:	Cell Biology, Ecology, Genetics, Plant Sciences and Psychology of Organism
Advance courses:	Virology, Ornithology and Oceanography
2008-2009	**Master in Science (1 year) & Bachelor Degree in Biology, Uppsala University. Uppsala, Sweden -Exchange year-**
Department:	Environmental Toxicology "Smile" Exchange program
Tutor:	PhD. Henrik Viberg
Projects:	"Repeated neonatal exposure to sucralose does not alter the expression of GAP-43, CaMKII, synaptophysin and tau proteins in cortex and hippocampus of mice" & "Repeated neonatal exposure to sucralose does not alter behavior in mice"
Advance courses:	Toxicology, Ecotoxicology, Behavioral Genetics and Global Challenges and a Sustainable Future
2004	**High School Diploma in Science,** **Maria Santisima High School. Caracas, Venezuela**

8.6 Presentation in meetings

Aiello Holden K.C., Castellani V., Bagnard D. & Bolz J. (14-18 July, 2012).
Sema3C acts as an attractive cue for migrating cortical interneurons. Abstract Nr. A- 471 – 0001 - 02385. Poster Nr. A23. Barcelona, Spain: **8th FENS Forum of Neuroscience** Supported by the Program for the Promotion of Equal Opportunities for Women and Men in Science (ProChance FSU)

8.7 Short research stays

I participated in the **"Young Investigator Training Program" (YITP)**, from the 8th FENS Forum of Neuroscience. Thank to this program I visited the group led by Dr. Eloisa Herrera in the Development and assembly of bilateral neural circuits groups from the Developmental neurobiology unit, Institute of Neuroscience; Alicante, Spain. This visit was funded by the International Brain Research Organization (IBRO) and the Western Europe Regional Committee (WERC). July-August 2012.

For getting trained in the preparation of the "conditioned media" and having feedback regarding my research, I visited Prof. Dr. Valerie Castellani in the group: Neurodevelopment and signaling, led by Dr. Valerie Castellani. Genetics and Molecular-Cellular Physiology Center, Claude Bernard Lyon1 University; Lyon, France. April 2012.

For getting feedback regarding my research I visited Prof. Dr. Dominique Bagnard in the group: Development of Novel Tumor Targeting Molecules, led by Dr. Dominique Bagnard. National Institute of Sanitary and Medical Research (INSERM U682), Strasbourg University; Strasbourg, France. April 2012.

I was trained by Dr. Christina Valkova in the confirmation of the protein production by the HEK cells lines, through western blot analysis. I also performed the photometric quantification of Semaphorin-AP activity in the Membrane traffic and Alzheimer Disease Unit, led by Christoph Kaether. Leibniz Institute for Age Research - Fritz Lipmann Institute; Jena Germany. May 2011.

8.8 Acknowledgment

Especially thank you Prof Dr. Jürgen Bolz. If I ever achieve half of your understanding and love for science, I would feel accomplished. Thanks for guiding me in the field of Developmental Neuroscience and especially into the fascinating world of Semaphorins. Thanks Prof. Dr. Jürgen Bolz for nurturing my project with great ideas and encouraging me to discuss my project with other Sema3C-research groups. But furthermore, I want to thank you for being a kind and comprehensive supervisor, regarding my situation as a foreigner student. "Vielen Dank" to the AG Bolz group where I was privileged to meet really inspiring and encouraging professionals. Work next to Dr. Judith Rudolph and Dr. Katrin Gerstmann prove me that being an excellent professional & achieving all personal goals -being thoughtful and keeping true to yourself- is possible.

I want to thank Prof. Dr. Dominique Bagnard and Prof. Dr. Valérie Castellani for receiving me in their laboratories and giving such a nice input to my project. Thanks to Dr. Christina Valkova for guiding me through the SDS-page and western blot performance. Dr. Annika Döding for providing me with such a perfect Western Blot. To Dr. André Steineke and Dr. Judith Rudolph for mentoring me and teaching me all the *in vitro* assays. Also to Falk Nietzsche and Céline Heng for performing some replicates for my assays.

I want to thank to the Exchange program ¡Ánimo, Chévere! ERASMUS Mundus and the FSU. Three years ago the idea of committing my days to research seemed utopian. Erasmus Mundus and the FSU made my academic goals possible. I also would like to thanks the Graduate Academy for their support during my final stage of the PhD.

These lines are dedicated to two of the most charismatic and science-lovers I have met: Dr. Katrin Gerstmann and Manuel Teichert. You are my best examples of a good scientist. Dr. Katrin Gerstmann: Without all our interesting discussions this work would not have been conceivable -thanks for going through my *crazy latina* manuscript. Furthermore, acknowledgements to Christine Raue and Elisabeth Meier, for excellent technical assistance and also thanks to Frau Behr for her kindness.

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Thanks to Elena and Alberto Salim Haubold for their assistance during the Erasmus Mundus application process. Exclusive cheers to Elena Salim Haubold that have been my big sister and tropical light in Germany. I wish to thank Phillipe Bernard, Jose Luis Pimentel, Diana Grillo, Mary Rodriguez, Mate Gutierrez, Sasha Salazar, Daniela Cerezo, Alejandra Aponte, Ana Karina Vivas, Laura Cano, Tania Ramirez, Miguel Angel Carmona, Javier Papa and Alejandra Geraldi. ALL of you thanks for your affection- for DREAMING big and have the courage to accomplish them.

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